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(54) Title: DIRECT SELECTION OF CELLS BY SECRETION PRODUCT

(57) Abstract

Cells can be labeled with products which they secrete and release in an efficient manner by coupling the cells at their surface to a specific binding partner for the product and allowing the product to be captured by the specific binding partner as it is secreted and released. The product-labeled cells can then be further coupled to suitable labels, if desired, and separated according to the presence, absence, or amount of product.

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<u>DIRECT SELECTION OF CELLS BY SECRETION PRODUCT</u>

10   <u>Technical Field</u>

The invention is in the field of analysis of
cell populations and cell separation. More particularly,
the invention concerns analysis and separation techniques
based on primary labeling of cells with their secreted

15   products through capture of these products by a specific
binding partner for the product anchored to the cell
surface.

<u>Background Art</u>

20   Numerous attempts have been made to analyze
populations of cells and to separate cells based on the
products which they produce. Such approaches to cell
analysis and separation are especially useful in
assessing those cells which are capable of secreting a

25   desired product (the "product"), or which are relatively
high secretors of the product. These methods include
cloning in microtiter plates and analysis of the culture
supernatant for product, cloning in agar and analysis by
methods for identification of the product of the

30   localized cells; the identification methods include, for
example, plaque assays and western blotting. Most
methods for analysis and selection of cells based upon
product secretion use the concept of physical isolation
of the cell, followed by incubation under conditions that

35   allow product secretion, and screening of the cell

locations to detect the cell or cell clones that produce
the product. For cells in suspension, after the cells
have secreted the product, the product diffuses from the
cell without leaving a marker to allow identification of
5   the cell from which it was secreted. Thus, secretor
cells cannot be separated from non-secretor cells with
this system.

In other cases, both secretor and non-secretor
cells may associate the product with the cell membrane.
10 An example of this type of system are B-cell derived cell
lines producing monoclonal antibodies. It has been
reported that these types of cell lines were separated by
fluorescence activated cell sorting (FACS) and other
methods reliant upon the presence of antibody cell
15 surface markers. However, procedures that analyze and
separate cells by markers that are naturally associated
with the cell surface may not accurately identify and/or
be used in the separation of secretor cells from non-
secretor cells. In addition, systems such as these are
20 not useful in identifying quantitative differences in
secretor cells (i.e., low level secretors from high level
secretors).

A method that has been used to overcome the
problems associated with product diffusion from the cells
25 has been to place the cell in a medium that inhibits the
rate of diffusion from the cell. A typical method has
been to immobilize the cell in a gel-like medium (agar),
and then to screen the agar plates for product production
using a system reliant upon blotting, for example western
30 blots. These systems are cumbersome and expensive if
large numbers of cells are to be analyzed for properties
of secretion, non-secretion, or amount of secretion.

Kohler et al. have described a system in which
mutants of a hybridoma line secreting IgM with anti-
35 trinitrophenyl (anti-TNP) specificity were enriched by

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coupling the hapten to the cell surface and incubating
the cells in the presence of complement. In this way,
cells secreting wild-type Ig committed suicide, whereas
cells secreting IgM with reduced lytic activity or not

5   binding to TNP preferentially survived. Kohler and
Schulman, Eur. J. Immunol. 10:467-476 (1980).

Other known systems allow the cells to secrete
their products in the context of microdroplets of agarose
gel which contain beads that bind the products, and

10   encapsulation of the cells. Such methods have been
disclosed in publications by Nir et al., Applied and
Environ. Microbiol. 56:2870-2875 (1990); and Nir et al.,
Applied and Environ. Microbiol. 56:3861-3866 (1990).
These methods are unsatisfactory for a variety of

15   reasons.

In the process of microencapsulation,
statistical trapping of numbers of cells in the capsules
occurs, resulting in either a high number of empty
capsules when encapsulation occurs at low cell

20   concentrations, or multiple cells per capsule when
encapsulation occurs at high cell concentrations. In
order to analyze and separate single cells or single cell
clusters by this technique, large volumes must be handled
to work with relatively small numbers of cells because of

25   the numbers of empty capsules and because of the size of
the microcapsules (50-100 μm). The large volume of
droplets results in background problems using flow
cytometry analysis and separation. In addition, the
capsules do not allow separation using magnetic beads or

30   panning for cell separation.

Various methods have been used to couple labels
to cell surfaces where the label is intended for direct
detection, such as a fluorochrome. For example, the use
of hydrophobic linkers inserted into the cell membrane to

35   couple fluorescent labels to cells have been described in

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PCT WO 90/02334, published 8 March 1990. Antibodies directed to HLA have also been used to bind labels to cell surfaces. Such binding results in a smaller dimension than the encapsulated droplets described above

5 and such cells can conveniently be used in standard separation procedures including flow cytometry and magnetic separations.

It has now been found that by anchoring a specific binding partner into the cell surface using an

10 appropriate coupling mechanism, products of the cells can be captured and cells sorted on the basis of the presence, absence or amount of product.

Disclosure of the Invention

15   The invention provides a method for convenient analysis and cell separation based on the products secreted by the cells. The cells are provided with a capture moiety for the product, which can then be used directly as a label. The binding of the product to the

20 capture moiety results in a "captured product." Alternatively, the cells are bound to the product via the capture moiety and can be further labeled via label moieties which bind specifically to the product and that are, in turn, labeled either directly or indirectly with

25 traditional labeling materials such as fluorophores, radioactive isotopes, chromophores or magnetic particles.

The labeled cells may then be separated or detected using standard cell sorting techniques based on these labels. Such techniques include flow cytometry,

30 magnetic separation, high gradient magnetic separation, centrifugation, and the like.

Thus, in one aspect, the invention encompasses a method to separate cells according to a product secreted and released by the cells, which method

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comprises effecting a separation of cells according to
the degree to which they are labeled with said product,
wherein labeling with the product is achieved by coupling
the surface of the cells to at least one capture moiety;
5   culturing the cells under conditions wherein the product
is secreted, released and specifically bound ("captured"
or "entrapped") to said capture moiety; and labeling the
captured product with the label moiety; wherein the
labeled cells are not lysed as part of the separation
10   procedure.

Another aspect of the invention is a
composition of matter which comprises cells capable of
capturing a product secreted and released by the cells
wherein the surface of the cells is coupled to a capture
15   moiety. Still another aspect of the invention is cells
and progeny thereof separated by the above-described
method.

Yet another aspect of the invention is a method
to label cells with a product secreted and released by
20   the cells, which method comprises coupling the surface of
the cells to a capture moiety, and culturing the cells
under conditions wherein the product is secreted and
released. The captured product may be separately labeled
by a label moiety.

25   An additional aspect of the invention is a
method of analyzing a population of cells to determine
the proportion of cells that secrete a varying amount of
product relative to other cells in the population, the
method comprising labeling the cells by the above-
30   described method, further labeling the cells with a
second label that does not label the captured product,
and detecting the amount of product label relative to the
second cell label.

Another additional aspect of the invention is a
35   method of determining a distribution of secretory

activity in a population of cells, the method comprising
labeling cells by the method described above (i.e.
coupling the surface of said cells to a capture moiety,
culturing the cells under conditions wherein the product

5   is secreted and released and exposing the cells to a
label moiety) and determining the amount of product per
cell by the amount of label moiety bound to the cell.

        Yet another additional aspect of the invention
is a method of determining a distribution of secreted

10   products and secretory activity for each secreted product
in a population of cells, the method comprising labeling
cells by the method described above by coupling the
surfaces of cells in the population with capture moieties
for each secreted product to be detected; culturing the

15   cells under conditions wherein the products are secreted
and released, labeling the secreted captured products,
with label moieties, wherein the label moiety for each
secreted capture product is distinguishable; and
determining the amount and type of product per cell.

20   Still another aspect of the invention is a kit
for use in the detection of cells that secrete a desired
product. The kit may contain a physiologically
acceptable medium which may be of varying degrees of
viscosity up to a gel-like consistency, said medium to be

25   used for cell incubation for the production of the
secreted product; a product capture system comprised of
at least one anchor moiety and at least one capture
moiety; at least one label moiety; and instructions for
use of the reagents, all packaged in appropriate

30   containers.

        Another aspect of the invention is a kit for
use in the detection and/or separation of cells that
secrete a desired product. The kit contains at least one
capture moiety which is a bifunctional antibody with

35   specificity for both the cells and the product and at

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least one label moiety. These reagents may, preferably, be placed in a single vial for simultaneous capture and labeling. Instructions for use of reagents should also be included. A physiologically acceptable medium of
5   varying viscosities or gel forming abilities may also be provided. The liquid phase may however be provided by the sample itself including but not limited to cell culture medium, blood and urine.

10   Brief Description of the Drawings

Figure 1 is a scheme for the introduction of biotinyl and palmitoyl groups onto Dextran.

Figure 2 is a scheme for the reaction of N-hydroxysuccinimide (NHS) esters with primary amino groups
15   in basic form.

Figure 3a and Figure 3b, respectively, are photocopies of traces of the fluorescence activated cell sorting analyses (FACScans) of binding of streptavidin to cells treated with biotinyldextran and
20   biotinylpalmitoyldextran.

Figure 4 are photocopies of traces of the FACScan results: a) unbiotinylated cells treated with streptavidin labeled with fluorisothiocyanate (ST-FITC) (negative control); b) cells treated with
25   biotinylpalmitoyldextran and then with ST-FITC; c) cells incubated with biotinyl-anti-$\alpha\beta_2$ microglobulin (β_2m) and treated with ST-FITC.

Figure 5 is a graph showing the titration of the binding of IgM to cells carrying conjugates of
30   biotinylpalmitoyldextran and capture antibodies.

Figure 6 are photocopies of traces of FACScan results of the capture with time of IgM on cells carrying capture antibody avidin-biotin conjugates. Panels (a), (b), (c), and (d) are the traces at 10 min, 30 min, 1 h,
35   and 2 h, respectively.

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Figure 7 are photocopies of traces of FACScan showing the effect of the concentration of methylcellulose in the medium on capture. Figures 7a and 7b show the capture of antibodies by cells incubated in

5   2.5% and 1% methylcellulose medium, respectively.

Figure 8 is a FACScan representation of labeled cells before and after separation based on capture of secreted antibodies in 2.5% methylcellulose containing medium. The cells are shown in the Figure 8a before

10   separation. Figure 8b shows the negative fraction after the separation. Figure 8c shows the positive fraction after the separation.

Figure 9a are photocopies of traces of FACScan results showing the effect of different added substances

15   in the culture medium during the secretion phase. Figures 9a, 9b, and 9c show the capture of product by anti-product antibodies on cells when the cells are incubated in culture medium, culture medium with 40% bovine serum albumin (BSA), and culture medium with 20%

20   BSA plus 20% gelatin, respectively.

Figures 10 and 11 are FACScan representations of labeled cells after labeling with capture antibody (10a, 11a), after the secretion phase (10b, 11b), and after magnetic separation, wherein (10c, 11c) are the

25   magnetic fraction, and (10d and 11d) are the nonmagnetic fraction.

Figure 12 shows the gating for Figures 13 and 14 of mouse spleen cells for FACS analysis. 12a is the forward scatter (FSC) v. side scatter (SSC) plot. 12b

30   shows propidium iodide (PI) v. fluorescence 2. The area enclosed by the lines shows the cells gated for further analysis (blast cells, living).

Figure 13 is a compilation of FACS analyses of FITC labeled cells. 13a is a scan of unlabeled cells.

35   13b is a scan of cells incubated with ST-FITC before

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biotinylation. 13c is a scan of cells labeled with
ST-FITC after biotinylation. 13d is a negative control
showing a scan of cells after biotinylated cells stained
with an antibody coupled to FITC specific for rat IgG
5   (GaRIgG-FITC) but which have not been exposed to the
avidinated catch antibody (rat) (catch-ab-avidin). 13e
is a scan of cells exposed to catch-ab and goat anti-rat
IgG-FITC (GaRIgG-FITC).

Figure 14 is a compilation of dot plots of FACS
10   analyses. In each instance, the abscissa represents the
amount of label staining for interferonγ (IFNγ) the
ordinate represents no information. Cells were labeled
with FITC labeled antidigoxigenin antibody detecting
digoxigenin-conjugated rat anti-mouse IFNγ antibody
15   (R46A2-DIG αDIG-FITC). 14a is the result obtained at
zero time incubation with catch antibody specific for
IFNγ (catch ab). 14b is the result obtained with a 5 min
incubation with the catch ab. 14c is the result obtained
with a 90 min incubation without the catch ab. 14d is
20   the result obtained with a 40 min incubation with the
catch ab. 14e is the result obtained when the cells with
catch ab are incubated in the presence of supernatant
obtained from cells secreting IFN-γ (IFNγ sup). 14f is
the result obtained with a 90 min incubation with catch
25   ab.

Modes of Carrying Out the Invention

The invention employs a mechanism for the
capture of products secreted from cells. The method
30   permits products secreted by eukaryotic and prokaryotic
cells or cell aggregates to be captured at the surface of
the cell. The captured product permits the cell to be
detected, analyzed, and if desired, sorted according to
the presence, absence, or amount of the product present.
35   The means of capture comprise a capture moiety which has

been anchored to the cell surface by a means suitable for the cell to be sorted. As used herein, the term "cell" or "cells" include cell aggregates; cell aggregates are groups of cells that produce a designated secreted

5   product and are known in the art, and include, for example, the islets of Langerhans. As used herein products which can be identified include any products secreted by the cells. Such products include, but are not limited to, cytokines like IFNγ, IL1, IL2, IL4, IL10,

10   IL12, TGFβ, TNF, GMCSF, and SCF, antibodies, hormones, enzymes and proteins.

The capture moiety may be coupled to the anchoring means (the "anchor moiety") optionally through a linking moiety, and may also include a linking moiety

15   which multiplies the number of capture moieties available and thus the potential for capture of product, such as branched polymers, including, for example, modified dextran molecules, polyethylene glycol, polypropylene glycol, polyvinyl alcohol, and polyvinylpyrrolidone.

20   For cells without cell walls, such as mammalian or other animal cells or cell protoplasts, suitable anchor moieties to the cell surface include lipophilic molecules such as fatty acids. Examples of suitable cell surface molecules include, but are not limited to, any

25   molecule associated with the cell surface. Suitable molecules include, but are not limited to, cell surface markers such as CD45 (pan leukocyte), anti-β2 microglobulin, CD3 (T cells (activating)), CD4, CD8, and other CD markers or cell adhesion molecules.

30   Alternatively, antibodies or other agents which specifically bind to cell surface molecules such as the MHC antigens or glycoproteins, could also be used. For cells which have cell walls, such as plant cells, fungi, yeast or bacteria, suitable anchor moieties include

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binding agents to cell wall components, including, for example, antibodies or lectins.

Specific binding partners include capture moieties and label moieties. The capture moieties are those which attach both to the cell, either directly or indirectly, and the product. The label moieties are those which attach to the product and may be directly or indirectly labeled. Specific binding partners include any moiety for which there is a relatively high affinity and specificity between product and its binding partner, and in which the dissociation of the product:partner complex is relatively slow so that the product:partner complex is detected during the labeling or cell separation technique.

Specific binding partners may include, but are not limited to, substrates or substrate analogs to which a product will bind. These substrates include, but are not limited to, peptides, polysaccharides, steroids, biotin, digitoxin, digitonin, and other molecules able to bind the secreted product, and in a preferred embodiment will include antibodies. When the capture moiety is an antibody it may be referred to as the "capture antibody" or "catch antibody." As used herein, the term "antibody" is intended to include polyclonal and monoclonal antibodies, chimeric antibodies, haptens and antibody fragments, bispecific antibodies and molecules which are antibody equivalents in that they specifically bind to an epitope on the product antigen.

Bispecific antibodies, also known as bifunctional antibodies, have at least one antigen recognition site for a first antigen and at least one antigen recognition site for a second antigen. Such antibodies can be produced by recombinant DNA methods or chemically by methods known in the art. Chemically created bispecific antibodies include but are not limited

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to antibodies that have been reduced and reformed so as
to retain their bivalent characteristics and antibodies
that have been chemically coupled so that they have at
least two antigen recognition sites for each antigen.
5   Bispecific antibodies include all antibodies or
conjugates of antibodies, or polymeric forms of
antibodies which are capable of recognizing two different
antigens. Antibodies can be immobilized on a polymer or
particle.

10   In the practice of the invention, the capture
moiety can be attached to a cell membrane (or cell wall)
by a variety of methods. Suitable methods include, but
are not limited to, direct chemical coupling to amino
groups of the protein components; coupling to thiols
15 (formed after reduction of disulfide bridges) of the
protein components; indirect coupling through antibodies
(including pairs of antibodies) or lectins; anchoring in
the lipid bilayer by means of a hydrophobic anchor
moiety; and binding to the negatively charged cell
20 surface by polycations.

            In other embodiments of the invention, the
capture moiety is introduced using two or more steps,
e.g., by labeling the cells with at least one anchor
moiety which allows the coupling of the capture moiety to
25 the anchor moiety either directly for instance by a
biotin/avidin complex or indiretly through a suitable
linking moiety or moieties.

            Methods for direct chemical coupling of
antibodies to the cell surface are known in the art, and
30 include, for example, coupling using glutaraldehyde or
maleimide activated antibodies. Methods for chemical
coupling using multiple step procedures include, for
example, biotinylation, coupling of TNP or digoxigenin
using, for example, succinimide esters of these
35 compounds. Biotinylation may be accomplished by, for

example, the use of D-biotinyl-N-hydroxysuccinimide.
Succinimide groups react effectively with amino groups at
pH values above 7, and preferentially between about pH
8.0 and about pH 8.5. Biotinylation may also be
accomplished by, for example, treating the cells with
dithiothreitol (DTT) followed by the addition of biotin
maleimide.

Coupling to the cells may also be accomplished
using antibodies against cell surface antigens
("markers"). Antibodies generally directed to surface
antigens may be required in the range of about 0.1 to 1
μg of antibody per 10^7 cells, however, this requirement
will vary widely in response to the affinity of the
antibody to the product and will need to be determined
empirically. Such a determination is well within the
skill of one in the art. Thus, the appropriate amount of
antibody must be determined empirically and is within the
skill of one in the art. This allows coupling to
specific cells on cell type specific marker expression.
For instance, classes of cells based such as T cells or
subsets thereof can be specifically labeled. As a
capture moiety, a bispecific antibody may be used which
has an antigen recognition site for the cell or an anchor
moiety placed thereon, and the product.

A capture moiety, particularly capture
antibodies should be selected based on the amount of
secreted product. For example, for cells which secrete
only a few molecules, a high affinity antibody should be
chosen so as to catch most of the secreted molecules.
Alternatively, in the case where the cell secretes many
molecules during the incubation time, a lower affinity
antibody may be preferred to prevent too early saturation
of the catching matrix. Determination of suitable
affinities for the level of proteins secreted are

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determined empirically and are within the skill of one in the art.

Cells carrying large amounts of N-acetylneuraminic acid on their surface as a constituent of their lipopolysaccharides bear a negative charge at physiological pH values. Coupling of capture moieties may be via charge interactions. For example, capture moieties bearing polycations bind to negatively charged cells. Polycations are known in the art and include, for example, polylysine and chitosan. Chitosan is a polymer consisting of D-glucosamine groups linked together by β-(1-4) glucoside bonds.

Another method of coupling capture moieties to the cells is via coupling to the cell surface polysaccharides. Substances which bind to polysaccharides are known in the art, and include, for example, lectins, including concanavalin A, solanum tuberosum, aleuria aurantia, datura stramonium, galanthus nivalis, helix pomatia, lens culinaris and other known lectins supplied by a number of companies including for example, Sigma Chemical Company and Aldrich Chemical Company.

In some embodiments of the invention, the capture moiety is coupled to the cell by hydrophobic anchor moieties to the cell membrane. Suitable hydrophobic anchor moieties that will interact with the lipid bilayer of the membrane are known in the art, and include, but are not limited to, fatty acids and non-ionic detergents (including, e.g., Tween-80). A drawback to attachment of the capture moiety to the cell via the insertion of an anchor moiety is that the rate of integration of the anchor moiety into the cell is low. Thus, high concentrations of the anchor moiety often are required. This latter situation is often uneconomical

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when the capture moiety is a relatively limited or
expensive substance, for example, an antibody.

The low yield of hydrophobic anchor moieties
that embed themselves in the membrane is relevant only
5   when these molecules are available in relatively limited
quantities. This problem may be overcome by using a
bridging system that includes an anchor moiety and a
capture moiety, wherein one of the moieties is of higher
availability, and wherein the two parts of the bridging
10   system have a high degree of specificity and affinity for
each other. For example, in one embodiment, avidin or
streptavidin is attached to the cell surface via a
hydrophobic anchor moiety, while the capture moiety is a
biotinylated anti-product antibody. In another
15   embodiment, the cell surface is labeled with digoxigenin
followed by bispecific antibodies having specificity for
both digoxigenin and the product. This approach can be
used with other pairs of molecules able to form a link,
including, for example, hapten with antihapten
20   antibodies, NTA with polyhistidine residues, or lectins
with polysaccharides. A preferred embodiment is one
which allows "amplification" of the system by increasing
the number of capture moieties per anchor moiety.

In one illustrative embodiment, a branched
25   dextran is bound to palmitic acid, thus providing a
multiplicity of available binding sites. The dextran is
in turn coupled to biotin and treated with avidin-
conjugated antibody specific for the product.

It is of course contemplated within the
30   embodiments of the invention that linker moieties may be
used between the anchor moiety and the capture moiety
when the anchor moiety is coupled in any fashion to the
cell surface. Thus, for example, an avidin (or
streptavidin) biotin linker moiety may link an antibody

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anchor moiety with a capture moiety. Bispecific antibody
systems may also act as linker moieties.

In order to analyze and, if desired, to select
cells that have the capability of secreting the product
5   of interest, cells modified as above to contain the
capture moiety are incubated under conditions that allow
the production and secretion of the product in a
sufficient amount to allow binding to and detection of
the cells that contain the captured product. These
10   conditions are known to those of skill in the art and
include, inter alia, appropriate temperature, pH, and
concentrations of salts, growth factors and substrates in
the incubation medium, as well as the appropriate
concentrations of gas in the gaseous phase. When it is
15   desirable to distinguish between high and low producer
cells, the time of incubation is such that product
secretion by the cells is still in a linear phase. The
appropriate conditions can be determined empirically and
such a determination is within the skill of one in the
20   art. Additionally, secretion by the cells can be
modified, that is upregulated, induced, or reduced using
a biological modifier. Suitable biological modifiers
include, but are not limited to, molecules and other
cells. Suitable molecules include, but are not limited
25   to, drugs, cytokines, small molecules, hormones,
combinations of interleukins, lectins and other
stimulating agents e.g. PMA, LPS, bispecific antibodies
and other agents which modify cellular functions or
protein expression.
30   Other cells include, but are not limited to,
direct cell to cell interactions such as between a tumor
and T cell and indirect cell to cell interactions such as
those induced by the proximity of other cells which
secrete a biological modifier. Suitable cells include,
35   but are not limited to, blood cells, peripheral bone

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marrow cells (PBMC) and various cell lines. The
biological modifiers can be added at any time but are
preferably added to the incubation medium.
Alternatively, the cells can be pretreated with these
5   agents or cells prior to the incubation step.

        The incubation conditions are also such that
product secreted by a producer cell is essentially not
captured by another cell, so distinguishing non-producing
cells from product producing cells, or high producers
10   from low producers is possible. Generally the incubation
time is between 5 minutes and ten hours, and more usually
is between 1 and 5 hours. The incubation medium may
optionally include a substance which slows diffusion of
the secreted product from the producer cell. Substances
15   which inhibit product diffusion in liquid media and that
are non-toxic to cells are known in the art, and include,
for example, a variety of substances that partially or
completely gel, including, for example, alginate, low
melting agarose and gelatin. By varying the viscosity or
20   permeability of the medium, the local capture by a
producing cell of certain sizes of secreted products can
be modulated. The molecular weight size exclusion of the
medium can be adjusted to optimize the reaction. The
optimal composition of the medium can be empirically
25   determined and is influenced by the cell concentration,
the level of secretion and molecular weight of the
product and the affinity of the capture antibodies for
the product. Such a determination is within the skill of
one in the art.
30   Preferably, the gels are solubilized after the
incubation to allow for the isolation of the cells or
groups of cells from the media by cell sorting
techniques. Thus, for example, the gels may be linked by
disulfide bonds that can be dissociated by sulfhydryl
35   reducing agents such as β-mercaptoethanol or DTT or the

gels may contain ionic cross-linkings, including for
example, calcium ions, that are solubilized by the
addition of a chelating agent such as EDTA.

In a preferred embodiment, during the secretion
5   phase, the cells are incubated in a gelatinous medium,
and preferentially the size limitation of penetration
into the gel prevents the product from substantially
entering the gel.

An alternative or addition to using a viscous
10   or gelatinous medium to prevent unspecific cell cross-
contamination is to provide a capture system for
capturing products not captured by the cell surface
capture system on the secreting cell. For example, this
technique can be used in the case where many cell types
15   produce a product or dead cells unspecifically release
large amounts of unwanted products or if no sufficient
diffusion barrier can be created between the cells. This
can be accomplished by adding to the medium surrounding
the cells beads (e.g. latex beads) conjugated to an
20   antibody product from the supernatant. Alternatively,
gels with immobilized antibodies or other moieties being
able to remove unbound product from the medium might be
employed. These trapping moieties are capable of
retaining these unwanted products or preventing them from
25   binding to the nonsecreting cells by binding to the non-
retained products. This "junk capture system" might
consist of immobilized into the gel matrix or it may be
attached to magnetic or other types of particles. The
location and catching characteristics of the junk capture
30   system should be adjusted so that sufficient product
molecules are specifically bound to the secreting cells
thus minimizing background on non-producing cells.

At the end of the secretion phase the cells are
usually chilled to prevent further secretion, and the gel
35   matrix (if any) is solubilized. This order may, of

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course, be reversed. The cells containing the captured product are then labeled with a label moiety. Labeling may be accomplished by any method known to those of skill in the art. For example, anti-product antibodies may be

5   used to directly or indirectly label the cells containing the product. The labels used are those which are suitable for use in systems in which cells are to be analyzed or sorted based upon the attachment of the label moiety to the product.

10   In other embodiments, capture moieties that do not contain captured product may be detected. This allows, for example, the isolation of cells that secrete high amounts of product by employing a negative separation method, i.e., detection of cells not highly

15   saturated with product. The cells can be labeled with other substances recognizing, including, but not limited to, cell surface markers, cell type, cellular parameters such as DNA content, cell status, or number of capture moieties.

20   The enumeration of actual capture moieties can be important to compensate for varying amounts of these molecules due to, for example, different conjugation potentials of the cells. It may be especially important for the isolation of rare cells to exclude cells with

25   decreased or increased capability for binding the product capture system, including the anchor and capture moieties.

          Analysis of the cell population and cell sorting based upon the presence of the label may be

30   accomplished by a number of techniques known in the art. Cells can be analyzed or sorted by, for example, flow cytometry or FACS. These techniques allow the analysis and sorting according to one or more parameters of the cells. Usually one or multiple secretion parameters can

35   be analyzed simultaneously in combination with other

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measurable parameters of the cell, including, but not
limited to, cell type, cell surface antigens, DNA
content, etc. The data can be analyzed and cells can be
sorted using any formula or combination of the measured

5   parameters. Cell sorting and cell analysis methods are
known in the art and are described in, for example, THE
HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, Volumes 1 to 4,
(D.N. Weir, editor) and FLOW CYTOMETRY AND CELL SORTING
(A. Radbruch, editor, Springer Verlag, 1992). Cells can

10   also be analyzed using microscopy techniques including,
for example, laser scanning microscopy, fluorescence
microscopy; techniques such as these may also be used in
combination with image analysis systems. Other methods
for cell sorting include, for example, panning and

15   separation using affinity techniques, including those
techniques using solid supports such as plates, beads and
columns.

        Some methods for cell sorting utilize magnetic
separations, and some of these methods utilize magnetic

20   beads. Different magnetic beads are available from a
number of sources, including for example, Dynal (Norway),
Advanced Magnetics (Cambridge, MA, U.S.A.), Immuncon
(Philadelphia, U.S.A.), Immunotec (Marseille, France),
and Miltenyi Biotec GmbH (Germany).

25   Preferred magnetic labeling methods include
colloidal superparamagnetic particles in a size range of
5 to 200 nm, preferably in a size of 10 to 100 nm. These
magnetic particles allow a quantitative magnetic labeling
of cells, thus the amount of coupled magnetic label is

30   proportional to the amount of bound product, and the
magnetic separation methods are sensitive to different
amounts of product secretion. Colloidal particles with
various specificities are known in the art, and are
available, for example, through Miltenyi Biotec GmbH.

35   The use of immunospecific fluorescent or magnetic

liposomes may also be used for quantitative labeling of
captured product. In these cases, the liposomes contain
magnetic material and/or fluorescent dyes conjugated with
antibody on their surfaces, and magnetic separation is
5   used to allow optimal separation between nonproducing,
low producing, and high producing cells.

The magnetic separation can be accomplished
with high efficiency by combining a second force to the
attractive magnetic force, causing a separation based
10   upon the different strengths of the two opposed forces.
Typical opposed forces are, for example, forces induced
by magnetic fluids mixed in the separation medium in the
magnetic separation chamber, gravity, and viscous forces
induced by flow speed of medium relative to the cell.
15   Any magnetic separation method, preferably magnetic
separation methods allows quantitative separation, can be
used. It is also contemplated that different separation
methods can be combined, for example, magnetic cell
sorting can be combined with FACS, to increase the
20   separation quality or to allow sorting by multiple
parameters.

Preferred techniques include high gradient
magnetic separation (HGMS), a procedure for selectively
retaining magnetic materials in a chamber or column
25   disposed in a magnetic field. In one application of this
technique the product is labeled by attaching it to a
magnetic particle. The attachment is generally through
association of the product with a label moiety which is
conjugated to a coating on the magnetic particle which
30   provides a functional group for the conjugation. The
product associated with the cell and coupled to a
magnetic label is suspended in a fluid which is then
applied to the chamber. In the presence of a magnetic
gradient supplied across the chamber, the magnetically
35   labeled cell is retained in the chamber; if the chamber

contains a matrix, it becomes associated with the matrix.
Cells which do not have or have only a low amount of
magnetic labels pass through the chamber.

The retained cells can then be eluted by
5   changing the strength of, or by eliminating, the magnetic
field or by introducing a magnetic fluid. The
selectivity for a captured product is supplied by the
label moiety conjugated either directly or indirectly to
the magnetic particle or by using a primary antibody and
10   a magnetic particle recognizing the primary antibody.
The chamber across which the magnetic field is applied is
often provided with a matrix of a material of suitable
magnetic susceptibility to induce a high magnetic field
gradient locally in the chamber in volumes close to the
15   surface of the matrix. This permits the retention of
fairly weakly magnetized particles. Publications
describing a variety of HGMS systems are known in the
art, and include, for example, U.S. Patent No. 4,452,773,
U.S. Patent No. 4,230,685, PCT application WO 85/04330,
20   U.S. Patent No. 4,770,183, and PCT/EP89/01602; systems
are also described in U.S. Serial No. 07/291,177 and in
U.S. Serial No. 07/291,176, which are commonly owned and
hereby incorporated herein by reference.

As seen from above, processes embodied by the
25   invention include the following steps:
    a.   Coupling an anchor moiety to the surface of
the cells suspected of secreting a product;
    b. coupling to the anchor moiety a capture
moiety which captures secreted product;
30   c.   incubating the cells with the coupled
capture moiety to allow synthesis and secretion of the
product under conditions whereby the product binds to the
capture moiety; and
    d.   labeling the captured product with a label
35   moiety.

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In addition, in other embodiments, the processes include labeling the cells that contain captured product, if any. Other embodiments may also include analyzing the cell population to detect labeled cells, if any, and if desired, sorting the labeled cells, if any.

The processes of the invention may be used to analyze and/or separate a variety of cell types. For example, it can be used to detect and select hybridoma cell lines that secrete high levels of antibodies.

An exemplary process for the selection of this type of hybridoma cell is the following. The cells are modified to contain a digoxigenin anchor moiety by coupling digoxigenin to the cell via a lipophilic anchor moiety or by chemical coupling. A capture moiety is linked to the cells via a rat anti-kappa or rat anti-lambda monoclonal antibody conjugated to anti-digoxigenin antibody or antibody fragments. The cells with the linked capture moiety are incubated to allow secretion of the monoclonal antibodies. Cells capturing the secreted product antibodies are labeled with the label moiety by incubation with rat anti-mouse IgG1 or IgG2a+b monoclonal antibody. An anti-class antibody that does not recognize the surface bound form of the product is advantageous when the expression product is naturally associated with the cell surface.

Selection of the high secretor cells is carried out in multiple rounds. Each separation process involves the use of a cell separation process, i.e., a quantitative magnetic separation system that distinguishes different levels of bound product, or a FACS. The cells having the highest labeling (eventually normalized on a cell to cell basis using further parameters) are sorted and expanded in culture again. Magnetic and FACS separation can be combined.

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FACS sorting is preferentially performed by
additionally labeling the cells for amount of capture
moiety using a different fluorochrome than that with
which the cells are originally labeled, then selecting
5   for cells with a high ratio of amount of product to
amount of antibody. Multiple rounds of separation using
high cell numbers of 10^7 to 10^{10} cells allows isolation
of rare genetic variants showing extraordinarily high
levels of production and genetic stability. In order to
10   avoid the selection of cells producing aberrant forms of
product, different label moieties may be used during the
different rounds of separation.

Using a similar approach, hybridomas with
defined specificity may also be detected and selected.
15   By employing a selection process on large cell numbers,
rare genetic variants with higher affinity or specificity
can be obtained. Class switch variants can be isolated
using different anti-class antibodies. Generally, this
approach can be used for the isolation of almost any kind
20   of variant of the antibodies with the desired
specificity.

The identification and isolation of genes
coding for a specific substance, and the isolation of
cells producing a specific protein, including specific
25   fusion proteins, cytokines, growth hormones, viral
proteins, bacterial proteins, etc., can also be
accomplished using the processes of the invention. For
example, if it is desirable to select for a cell
producing a specific protein, the cells can be
30   genetically modified by the introduction of an expression
vector that encodes the protein of interest in a secreted
form. The cells are modified by the introduction of a
product capture system, including an anchor moiety and a
capture moiety specific to the product, and the cells are
35   grown under conditions that allow product secretion. The

cells containing the captured product are labeled, and subjected to one or more rounds of separation based upon the presence of label.

Such separation of cells expressing an artificially introduced gene resulting in a secreted product is particularly useful in gene therapy methods where patient cells are removed from the body and transformed with the gene resulting in the secretion of a certain product (e.g., a cytokine). The transformed cells are then isolated from non-transformed cells before being returned to the patient. At present the method used is cumbersome and time-consuming. The cells are transformed not only with the gene expressing the protein of interest but also with a gene expressing a marker protein. Current techniques utilize β-galactosidase as the marker protein thus, the cells must be cultured in the presence of X-gal and those cells which turn blue are hand-picked and returned to the patient. Not only is this laborious but it results in serious extensions of time prior to treatment of these often gravely ill patients. With the method described herein, the transformed cells can be separated soon after transformation and returned immediately to the patient. The separation can also be based on secretion of the protein of interest rather than a marker protein so as to ensure the cells are transformed and expressing the protein of interest.

The process of the invention may also be used to simultaneously analyze qualitative and quantitative secretion patterns in complex cell mixtures such as, for example, mixtures containing white blood cells, bone marrow cells, suspended tumor cells, or tissue cells. In this case, the cells in the mixture would be labeled with cell specific markers, and would also be labeled with capture moieties for the products to be detected. The

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cells could also be labeled with bispecific antibodies containing at least one antigen recognition site for the specific cell marker and at least one antigen recognition site for the products to be detected.

5   After the secretion phase, the cells would be subjected to multiparameter analysis as used in flow cytometry and/or image analysis, and the results analyzed with multi-dimensional statistical methods known in the art, and used in the analysis of flow cytometry and image

10   analysis data. If the analysis is to determine cells specifically reactive with a biological modifier the cells to be analyzed can be exposed to these biological modifiers prior to and/or during the incubation period prior to analysis by flow cytometry or image analysis.

15   Methods such as these are potentially of value for various diagnostic applications in medicine, for example, for measuring levels and types of interleukin production in various cell populations, and for measuring growth factor release in tumor cell populations.

20   It is contemplated that the reagents used in the detection of secretor cells of desired products may be packaged in the form of kits for convenience. The kits would contain, for example, optionally one or more materials for use in preparing gelatinous cell culture

25   medium, the medium to be used for cell incubation for the production of the desired secreted product; a product capture system comprised of anchor and capture moieties; a label moiety; and instructions for use of the reagents. All the reagents would be packaged in appropriate

30   containers.

           The kit may also be formulated to include the following. In this case all the reagents are preferably placed in a single vial to which the cells are added. At least one antibody which is bispecific for a particular

35   cell surface structure or anchor moiety and the product.

At least one label moiety and, optionally, biological modifiers.

The label moiety may be a fluorochromated anti-product antibody, which may include, but is not limited
5   to, magnetic bead conjugated, colloidal bead conjugated, FITC, Phycoerythrin, PerCP, AMCA, fluorescent particle or liposome conjugated antibodies. Alternatively the label moiety may be any suitable label including but no0t limited to those described herein.
10   Optionally, the kit may include physiologically acceptable buffer. Such buffers are known in the art and include, but are not limited to, PBS with and without BSA, isotonic saline, cell culture media and any special medium required by the particular cell type. Buffers
15   might be used that reduce cross-labeling and increase the local product concentration around the cells. Buffers may include agents for increasing viscosity or decreasing permeability. Suitable agents are described herein. The viscosity of the medium can be reduced before analysis by
20   any method known in the art including, but not limited to, dissolution in a physiologically acceptable buffer, dissolving heat, EDTA, and enzymes. In the absence of added medium cells already suspended in a medium may be directly added to the vial. Suitable cell suspensions
25   include but are not limited to cell lines and biological samples. Biological samples include, but are not limited to, blood, urine and plasma.
        Additional structures may be added for catching unbound product to reduce cell cross-contamination
30   thereby reducing the diffusion of products away from the producing cells. These include, but are not limited to, anti-product antibody immobilized to gel elements, beads, magnetic beads, polymers.
        Biological modifiers may also be added to the
35   buffer or medium to induce specific secretion.

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Additional label moieties such as antibodies (magnetically or fluorescently labeled) are also present, including, but not limited to anti-cell surface antibodies to identify cell types, propidium iodide to

5   label dead cells, and magnetic beads to label certain cell types.

In this embodiment, all materials can be placed in a single container such as a vial and the cell sample added. The contents are incubated to allow secretion of

10   a product and subsequent capture of the product and binding of the label moiety to the product. The cells which have secreted and bound product can then be separated and/or analyzed based on the presence, absence or amount of the captured product. Separation may be

15   done by any of the methods known in the art, including, but not limited to, simple dilution, erythrocyte lysis, centrifugation-washing step, magnetic separation, FACS and Ficoll separation. The analysis of the cells may be performed by a variety of methods, including, but not

20   limited to, FACS, image analysis, cytological labeling, and immunoassay.

As shown below, in the examples, cells secreting designated products can be identified and sorted within minutes of incubation in the presence of

25   the specific binding partners. Thus the kits described are suitable for use in diagnostic applications. For instance, suitable diagnostic applications include, but are not limited to, immune disregulations, genetic defects and cancer classification.

30   The examples described below are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the

35   art.

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Examples
Example 1

The purpose of this example was to separate
living cells that secrete a given product from a mixture
of externally identical cells. The B.1.8. hybridoma cell
line and the X63. Ag 8 6.5.3. myeloma cell line were used
as the test system. About 60% of B.1.8. cells secrete
IgM; the myeloma line secretes no protein. The secreting
cells were to be separated from B.1.8. and from mixtures
of B.1.8. with Ag 8 6.5.3. cells. To achieve this, a
procedure was developed to capture, on the cell surface,
a product secreted by the cell, hold the product on the
cell surface, and thus label the cell in question. The
capture antibodies were attached in two steps: 1)
biotinylation of the cells; and 2) attachment of the
capture antibody through an avidin-biotin coupling
reaction. The labeled cells were then separated from
cell mixtures.

Biotinylation of the Cell Surface
with Biotinylpalmitoyldextran

The objective was the synthesis of an anchor
moiety which is a large macromolecule with biotin groups
and fatty acid groups that was to embed itself in the
cell membrane.

Synthesis of a Hydrophobic Biotin

A dextran with a molecular weight of 3 x 10^6
g/mole was used as the carrier molecule. In order to be
able to couple both biotin groups and the fatty acid
group to the polysaccharide, reactive primary amino
groups first were introduced into the dextran.

Biotinyl groups and a palmitoyl group were then
to be introduced to the amino groups of proteins by
somewhat modified methods such as those used for coupling
biotin and fatty acid esters. Figure 1 shows the scheme

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for the introduction of biotinyl and palmitoyl groups
onto Dextran.

Synthesis of an Aminodextran

Amino groups were introduced into Dextran
5   molecules by activation with carbodiimidazole and
reaction with diaminohexan using standard methods.
Aminodextran was obtained from Sigma Corp. and from
Molecular Probes (Oregon). An aminodextran with 165 $\pm$ 20
amino groups per molecule of 3 x 10^6 g/mole was obtained.
10 Polymerization of dextran occurs as a side reaction. The
yield of unpolymerized product amounted to 94% of the
starting dextran.

A method described by Dubois was used to
determine dextran concentrations. 5 μl of an 80%
15 solution of phenol in water was placed in a test tube
with 100 μl of the dextran solution to be determined. 1
ml of concentrated sulfuric acid was pipetted quickly
into this mixture. After 10 minutes, the formulation was
placed in a water bath at 30°C for 10 minutes longer.
20 The dextran concentration was found by measuring the
extinction at 480 nm.

Synthesis of Biotinylaminodextran

The introduction of biotinyl groups onto the
dextran was accomplished using D-biotinyl-N-
25 hydroxysuccinimide as the biotinylation reagent.
Succinimide groups react effectively with amino groups at
pH values above 7. Figure 2 is a scheme for the reaction
of N-hydroxysuccinimide esters with primary amino groups
in basic form. The corresponding N-hydroxysuccinimide
30 (NHS) esters were used for introducing both the biotinyl
groups and the palmitoyl groups in the dextran. In this
Figure, R' stands for dextran, and R stands for either a
biotinyl group or for a palmitoyl group.

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Synthesis of Biotinylpalmitoyldextran

Palmitic acid groups were coupled to the
biotinylated dextran. The reaction was carried out by a
slightly modified procedure for coupling palmitoyl groups
to antibodies (Huang et al., J. Biol. Chem. 255:8015-8018
(1980)). The coupling occurs similarly to the preceding
biotinylation by nucleophilic attack of the amino groups
of the dextran on the NHS ester of palmitic acid.

Biotinylation of Cells with Biotinylpalmitoyldextran

The ability of the lipopolysaccharide,
biotinylpalmitoyldextran, to bind to cells and thereby
biotinylate the cell surface was tested on human
lymphocytes and compared with the binding of
biotinylaminodextrans lacking palmitoyl groups.

The cells were centrifuged out at 20°C and
incubated for 10 minutes at 37°C with 1 mg/ml of either
biotinyldextran or biotinylpalmitoyldextran in PBS (100
μl for 10^7 cells). 1 ml of PBS 1% BSA (PBS/BSA) was then
added, and after 3 minutes the cells were washed on ice
in 14 ml of PBS. The treated cells were taken up in PBS
0.03% sodium azide (PBS/NaN$_3$).

Biotinylation of the cells by biotinyldextran
or biotinylpalmitoyldextran was monitored by labeling of
the cells with streptavidin-FITC (ST-FITC). More
specifically, the treated cells were washed and taken up
in 100 μl of PBS/10^7 cells. 1 μl of 100 μg/ml ST-FITC in
PBS was added and the mixtures were incubated for 5
minutes on ice. The cells were then washed, taken up in
1 ml of PBS/BSA per 10^7 cells, and the intensity of
fluorescence was measured in the FACScan as a measure of
biotinylation.

The results of the FACScans of binding of
streptavidin to cells treated with biotinyldextran and
biotinylpalmitoyldextran are shown in Figure 3a and
Figure 3b, respectively. As seen from the results, cells

incubated with biotinyldextran did not bind ST-FITC.   In
contrast, cells incubated with biotinylpalmitoyldextran
bound large amounts of the ST-FITC.

5   A comparison was made between the amount of ST-
FITC bound by cells labeled with biotinyl antibodies
directed towards β_2 microglobulin (B_2m) and the
biotinylpalmitoyldextran labeled cells.   The antibody
used was $\alpha\beta_2$m, an antibody that binds to β_2m.   Figure 4
shows traces of the FACScan results: a) unbiotinylated
10   cells treated with ST-FITC (negative control); b) cells
treated with biotinylpalmitoyldextran and then with ST-
FITC; c) cells incubated with biotinyl-$\alpha\beta_2$m and treated
with ST-FITC.   The results in Figure 4 indicate that
cells labeled with biotinylpalmitoyldextran are able to
15   bind more streptavidin to the cell surface than an $\alpha\beta_2$m-
biotin conjugate.

      While antibody labeling of the cell reaches
saturation, labeling by biotinylpalmitoyldextran
increases linearly with the concentration of the labeling
20   reagent.   However, the labeling is limited by injury to
the cells when the concentrations of reagent are too
high.   When biotinylation of the cells was with about 1
mg/ml of biotinylpalmitoyldextran for 10 minutes at 37°C,
no change of the cell surface was observed under the
25   microscope; the light-scattering properties of the cell
surface, which were measured in the FACScan with forward
and lateral scattered light, were unchanged compared to
untreated cells.   The treated cells maintained viability
and could be cultured again.

30   <u>Coupling of Capture Antibodies to Biotinylated Cells</u>

      Capture antibodies were coupled to cells
biotinylated with biotinylpalmitoyldextran via an avidin-
biotin bridge.   In order to accomplish this, the capture
antibodies were conjugated with avidin, and the
35   conjugates reacted with the biotinylated cells.

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Two antibodies, rat anti-mouse IgM (R33.24.12) and mouse kappa against mouse lambda (LS136) against various epitopes on mouse IgM (lambda) were coupled to avidin.

5   Avidin is a basic protein with several reactive amino groups. Succinimydyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) was used to couple avidin to the capture antibodies. SMCC is a bivalent linker molecule whose maleimide group reacts selectively

10   with thiols and whose succinimydyl group reacts selectively with primary amines. The capture antibody was reduced with DTT. DTT is a mild reducing agent that under suitable conditions reduces 1-4 disulfide bridges of an IgG molecule to thiols without destroying the

15   antigen-binding site. A reactive maleimide group was introduced on the amino groups of avidin with SMCC. The maleimide group of avidin was reacted with the SH groups of the reduced antibody. Avidin and capture antibodies were joined in this way through a cyclohexane bridge.

20   More specifically, 1.5 μl of a 1 molar solution of DTT was added to 1 mg of antibody of the IgG class in 200 μl of PBS containing 5 mM EDTA (PBS/EDTA). After reaction for 1 hour at room temperature, the reduced antibody was placed on a Sephadex PD10 column and eluted

25   in 1 ml of PBS/EDTA. The number of thiol groups introduced per antibody molecule was determined. The desirable range is about 2-6 thiol groups per antibody molecule.

Concomitantly, 1 mg of avidin was dissolved in

30   100 μl of carbonate buffer pH = 9.4 and 125 μg of SMCC in 7.5 μl of DMSO were added. After 1 hour at room temperature, the protein was purified on a Sephadex PD10 column and taken up in 500 μl of PBS/EDTA.

1 mg of the reduced antibody in 1 ml of

35   PBS/EDTA was combined with 400 μg of the activated avidin

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in 200 μg of PBS/EDTA and allowed to stand overnight at
4°C. The reaction was stopped by adding 5 μl of 1 M
N-ethylmaleimide.

Coupling of Avidin-labeled Capture Antibodies to
5   Biotinylated Cells

A mixture of myeloma and hybridoma cells was
used. B.1.8. hybridoma cells that secrete IgM and
nonproducing X63. Ag 8 6.5.3 myeloma cells were grown at
37°C in an atmosphere saturated with water vapor. The
10 culture medium contained RPMI and 5% fetal calf serum
(FCS), 100 IU/ml of penicillin, and 0.1 mg/ml of
streptomycin.

The cell mixture was biotinylated with
biotinylpalmitoyldextran using the conditions described
15 above.

In order to couple the antibody-avidin
conjugates to the biotinylated cells, the biotinylated
cells, after washing in PBS/1% BSA, were incubated with
an avidin-capture antibody conjugate. 1 μl of a solution
20 of 1 mg/ml of capture antibody-avidin conjugate in PBS
was added to 10^7 biotinylated cells in 100 μl of
PBS/NaN$_3$. After 10 minutes on ice, the biotin groups
were saturated with capture antibody, and the cells were
loaded with capture antibodies.

25 In order to detect the presence of the avidin-
antibody complexes on the cell surface, a fluorescent
anti-antibody was used, and the fluorescent labeling
detected by FACScan. The labeling of cells corresponded
approximately to the labeling of biotinylated cells with
30 fluorescent streptavidin, performed in the same study. A
uniform labeling of the cell population was observed; all
of the cells carried about the same amounts of capture
antibodies on their surfaces.

Testing the Functionality of Capture Antibodies on the
35 Cell Surface

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About 10^7 cells provided with capture antibodies were incubated on ice (so that they secreted no protein) in 100 μl PBS/BSA, with various concentrations of mouse IgM, which is captured by the

5   capture antibodies. After 5 minutes of incubation, the cells were washed and the captured IgM was detected on the cell surface using R-PE conjugate as the antibody label. Detection was in the FACScan. Figure 5 shows the titration curve. In the figure, the fluorescence of the

10   cells (mean) is plotted against the IgM concentration with which the cells were incubated. These results show that the capture antibody on the cell surface still has intact binding sites. The sensitivity of the capture antibodies to low IgM concentrations in the medium is

15   also recognizable. The titration curve illustrated was obtained using R33.24.12 as capture antibody. The capture antibody LS136 was used for the capture experiments shown later. The latter showed somewhat higher sensitivity to low IgM concentrations in the

20   medium.

Capture of Secreted IgM

A mixture of biotinylated B.1.8. and X63 cells was conjugated with capture antibodies and was kept under a 7.5% CO_2 atmosphere at 37°C for various lengths of time

25   in medium. The IgM captured on its surface was then detected by an antibody label.

Figure 6 shows the resulting labelings as FACScan illustrations: duration of capture test (6a) 10 min; (6b) 30 min; (6c) 1 h; and (6d) 2 h. Two

30   populations can be differentiated after 30 min, which have captured different amounts of IgM. The difference between the two populations disappears after lengthy incubation because of IgM given off to the medium by the secreting cells, which is taken up by the capture

35   antibodies on the nonsecreting cells.

Capture of Secreted IgM Using a Diffusion Inhibitor

It can be seen from the illustration above that the less strongly labeled cell population also takes up IgM rapidly on its surface. This background labeling

5   comes from secreted IgM in the culture medium that has not been captured by the capture antibodies on the secreting cells. If the capture experiment is carried out in a more viscous medium, this background labeling can be reduced. Culture medium with 2.5% methylcellulose

10   was used; this medium shows a gel-like consistency.

The cells loaded with capture antibodies were mixed in culture medium with 2.5% methylcellulose or 1% methylcellulose. It was unnecessary and superfluous to wash the cells; capture antibody-avidin conjugate not

15   bound to the cells does not interfere. 2 ml of medium was used for 10^7 cells. To bring the methylcellulose properly into solution, it was admixed with the culture medium one day previously. The medium was preheated to 37°C, the cells were added and were incubated for 25 to

20   45 minutes at 37°C with 7.5% CO_2. Under these conditions, the hybridoma cells secreted their product. After the incubation time, the high-viscosity medium was diluted with 45 ml of cold PBS/BSA. The cells were centrifuged out at 4°C and taken up in 100 to 500 μl of

25   PBS/BSA. Remainders of methylcellulose gave the cell suspension an elevated viscosity; neither the cells nor the subsequent labeling steps were harmed by this.

Figure 7 shows the effect of the concentration of methylcellulose in the medium on capture. The cells

30   in this experiment produced IgM for 35 minutes in 2.5% methylcellulose, and were then washed and labeled with R33.24.12. R-PE. Figures 7a and 7b show the capture of antibodies by cells incubated in 2.5% and 1% methylcellulose medium, respectively. The results

35   indicate that the secreting and non-secreting cells were

successfully distinguished based on capture in the 2.5%
methylcellulose medium.

Double Labeling

5   To show that the cells carrying IgM on their
surface after the capture experiment described above
actually are cells secreting IgM, the cells were labeled
red on the surface after the capture experiment with
R33.24.12. R-PE (visible in the FACScan as Fluorescence
2.), fixed, and labeled green in the cytoplasm with
10   R.33.24.12.-FITC (visible in the FACScan as Fluorescence
1.). The cells labeled twice in this way were examined
under the microscope and in the FACScan.

The fact that the cells carrying IgM on their
surface after the capture experiment are secreting cells
15   was illustrated by this double labeling as a two-
dimensional representation of Fluorescence 1 and 2 in the
FACScan. B.1.8. cells after the capture experiment were
labeled red on their surface relative to the captured IgM
(visible in the FACScan as Fluorescence 2), and were then
20   fixed and labeled green in the cytoplasm relative to IgM
(visible in the FACScan as Fluorescence 1). All of the
surface-labeled cells are also labeled in the cytoplasm.

The results indicated that all of the cells not
producing IgM also belonged to the cell population that
25   were not surface-labeled. The cytoplasm-positive cells
were divided into two fractions; on the one hand, a
fraction of cells labeled both on the surface and in the
cytoplasm. These were apparently secreting cells. On
the other hand, a cell fraction was labeled in the
30   cytoplasm but not on the cell surface. Since this
population could not be separated by a Ficoll gradient
(carried out just before fixation), they were not dead
cells. Some of the cells in this population were also
not labeled as intensely in the cytoplasm as the
35   secreting cells. The broader dispersion of this fraction

compared to the two other cell populations was also
striking. These cells produced IgM but apparently lost
the ability to secrete this protein. The double-labeled
cells were observed under the microscope as a control.

5   This examination showed conformity with the outcome of
the FACScan representation.

Cell Separation of the MACS

        After the capture of secreted IgM with a 1:1
mixture of about 10^7 B.1.8. and X63 cells, separations

10 were carried out with the magnetic cell sorting system
(MACS), using magnetic particles that bind to the
captured IgM on the cell surface. The MACS system and
magnetic particles were from Miltenyi Biotec GmbH
(Germany).

15   A mixture of IgM-secreting and nonsecreting
cells was provided with the matrix for capturing secreted
IgM developed in this work and was kept at 37°C in an
atmosphere of 7.5% CO_2 for 25 minutes in 5 ml of culture
medium with 2.5% methylcellulose. The cells were washed

20 in 45 ml of PBS/BSA. The pellet was treated with a
remainder of methylcellulose of gel-like consistency. It
was taken up in 500 μl of PBS/BSA and 5 μl of rat anti-
mouse IgM magnetic beads (Miltenyi Biotec GmbH) were
added. After 5 minutes on ice, 10 μg/ml of R-PE-coupled

25 R33.24.12 antibody was added and the mixture was kept on
ice for 5 min longer.

        About 10^7 cells pretreated in this way were
placed on a type Al separating column in the MACS
(Miltenyi Biotec GmbH) and the negative fraction was

30 eluted with 10 ml of PBS/BSA at 5°C. After removing the
column from the magnetic field, the positive cell
fraction was eluted in 10 ml of PBS/BSA.

        Cells surface-labeled red relative to IgM are
shown in Figure 8 before and after the separation. The

35 cell suspension prior to separation contained 58.8%

unlabeled and 41.2% labeled cells. After the separation,
the negative fraction contained 89% unlabeled and 11%
labeled cells. The positive fraction contained 23%
unlabeled and 77% labeled cells. When the concentration

5   of positive cells after separation is calculated with the
formula: concentration factor = (% pos. cells in pos.
fraction * % neg cells in original cell mixture) / % pos.
cells in the original fraction * % neg cells in pos.
fraction), a concentration factor of 4.8 is obtained.

10   After the separation, the cells could not be
labeled with propidium iodide, a dye that selectively
labels dead cells, and could again be cultured. The
vitality of the separated cell fractions was checked
under the microscope one week after the separation. The

15   loss of cells during the separation process was not
determined; no relevant losses of cells normally occur in
separations in the MACS.

             Figure 8 is a FACScan representation of labeled
cells before and after separations. After capturing

20   secreted IgM, the cells were labeled relative to IgM on
the surface and were separated in the MACS with magnetic
particles relative to IgM. The cells are shown in the
Figure 8a before separation. Figure 8b shows the
negative fraction after the separation. Figure 8c shows

25   the positive fraction after the separation. If the cells
to the right of the broken line are considered to be
labeled and those on the left of it to be unlabeled, the
cell fractions contained the following amounts of labeled
and unlabeled cells:

30

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	% neg.	% pos.
Cells before separation	58.8	41.2
Negative fraction after separation	89	11
Positive fraction after separation	23	77

The studies described above included the following general techniques.

Antibody Labeling of Cells

The cells were taken up in PBS/BSA and pelleted by centrifugation. The supernatant was removed by suction, and the pellet resuspended in the antibody labeling solution. 100 μl of labeling solution containing 10-100 μg/ml of antibody in PBS/BSA 0.1% NaN_3 was used per 10^7 cells. The coupling reaction was incubated 5 minutes on ice. The cells were then washed.

Ficoll Gradient Centrifugation

Ficoll gradient centrifugation was used to remove dead cells. The cell suspensions were carefully underlayered with 5 ml of Ficoll (Pharmacia LKB, Uppsala, Sweden) and were then centrifuged at 2500 rpm at room temperature. Living cells remained resting on the Ficoll cushion and were removed by suction.

Cytoplasm Labeling

0.5% saponin and 10 μg/ml of labeling antibody were added to the fixed cells in PBS/BSA. Saponin produces reversible channels about 10 nm in diameter in the cell membrane, so that the antibodies can penetrate into the cells. After a reaction time of 1 hour the cells were taken up in PBS/BSA 0.5% saponin (1 ml/10^6

cells). After 30 minutes, the cells were washed and
taken up in saponin-free PBS/BSA.

Antibody Used

5   R33.24.12., a monoclonal rat anti-mouse
antibody, coupled both to R-PE and to fluorescein, was
obtained from stocks of the Immunobiological Department
of the Genetic Institute of Cologne. The optimal
labeling concentrations were titrated. The R-PE
10   conjugate of this antibody was used to label the captured
IgM on the surface of secreting cells, and the
fluorescein conjugate was used for cytoplasm labeling.
LS136, a mouse IgG kappa against mouse lambda is used as
the capture antibody (the IgM to be captured is of the
15   lambda allotype). LS136 likewise originates from the
internal production of the Immunobiological Department.

Example 2

This example demonstrates the effect of
carrying out the secretion phase in a gelatinous medium
20   as compared to a high viscosity medium on the capture of
secreted product by cells containing a biotin anchor
moiety linked via avidin to the capture moiety.

Chemical biotinylation of cells using NHS-LC-Biotin

25   A mixture of B.1.8. and X63 cells was
chemically biotinylated by the following procedure. Cell
suspensions containing 10^7 to 10^8 cells were centrifuged,
the supernatant removed, and the pellet resuspended in a
solution of 200 μl PBS pH 8.5, containing 0.1 to 1 mg/ml
30   NHS-LC-Biotin (Pierce, Rockford, Illinois, U.S.A.).
After incubation for 30 minutes at room temperature the
cells were washed two times extensively with 50 ml
PBS/BSA. Labeling with avidin conjugate was within 24
hours of the biotinylation.

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Linkage of the biotinylated cells to capture antibodies
with avidin

     The cells biotinylated by reaction with NHS-LC-
Biotin were labeled with an avidin conjugate of LS136
5   (concentration of 30 μg/ml) for 30 minutes on ice and
washed.

Secretion and product capture in gelatinous medium and in
high viscosity medium

10   The biotinylated-avidin treated cells were
incubated 1 hour at 37°C under 7.5% CO_2 in three
different media, washed and labeled with a fluorescein
conjugate of R 33.24.12 (10 μg/ml) for 10 minutes on ice,
washed and analyzed using flow cytometry (FACScan) for
15   determination of the amount of bound R 33.24.12.
R 33.24.12 is a fluorescein conjugate of the anti-product
antibody. The three different media used during the
incubation were: (1) cell culture medium, RPMI, 5% FCS;
(2) RPMI, 5% FCS, supplemented with 40% BSA (Fluka,
20   Switzerland); and (3) RPMI, 5% FCS, supplemented with 20%
BSA and 20% gelatin (Type B from bovine skin approx. 225
bloom, Sigma Chemical Co.) as a gelatinous diffusion
inhibitor. The results are shown in Figures 9, 10, and
11.
25   Figure 9a shows the distribution of labeling of
the cells incubated in RPMI, 5% FCS (i.e., without a
diffusion inhibitor). The entire cell population is
shifted towards higher fluorescence, thus no separation
in distinct cell populations can be resolved. Figure 9b
30   shows the distribution of labeling of cells incubated in
RPMI, 5% FCS supplemented with 40% BSA. This BSA medium
is a high viscosity diffusion inhibitor. Compared to
Figure 9a, it shows that incubation in this medium led to
less background labeling. Figure 9c shows the
35   distribution of labeling of the cells incubated in RPMI,

5% FCS, supplemented with 20% BSA and 20% gelatin. Using this medium two cell populations, secretors and nonsecretors can be identified. Compared to the cells incubated in the other two media as indicated in Figures

5 9a and 9b, the amount of fluorescence on the secretor population is significantly increased.

This example shows that while a viscous medium such as a high BSA medium will decrease capture of secreted product by non-producer cells, incubation during

10 secretion in a gelatinous medium results in significantly increased labeling of the producer cells with a concomitant lowering of capture non-producer cells. This amplification effect on capture allows the labeling of cells producing lower levels of product and/or allows the

15 use of lower affinity antibodies for the capture of the secreted product. The gelatinous medium appears to result in an increased concentration of the product in the vicinity of the secreting cells while not inhibiting the speed of the capture reaction. When gelatinous media

20 with a cutoff limit lower than the molecular weight of the product is used in the medium, the secreted molecules may concentrate in the gap between cell and medium, resulting in higher local concentrations and more efficient labeling of the secreting cells.

25

Cell Separation using MACS

A mixture of B1.8 and X63 cells were chemically biotinylated and labeled with LS136-avidin, as described above. A control sample was taken and stored on ice.

30 The remaining cells were allowed to secrete for 1 hour in 6 ml gelatinous RPMI medium containing 23% gelatin, 18% BSA and 5% FCS. The gel was quickly dissolved in 20 ml of 42°C PBS, followed by the rapid addition of 30 ml ice-cold PBS and washing in a cooled centrifuge. The cells

35 and the control sample were labeled for 10 minutes on ice

with rat anti-mouse IgM microbeads (Miltenyi Biotec GmbH, labeled with goat anti-mouse fluorescein (SBA, Birmingham, Alabama) and washed once. The cells were then separated on an A2 column using a MACS magnetic cell

5   sorter. Separation was performed according to the manufacturer's instructions. The control sample, unseparated sample, and magnetic and non-magnetic fractions were analyzed by Flow Cytometry (FACScan) (Becton Dickinson, San Jose, CA, USA).

10   Figure 11a shows the fluorescence distribution of the control sample. As seen in the figure, almost no detectable surface labeling was detected on the cells (0.6% in area between dotted lines (positive window)). Figure 11b shows the fluorescence distribution after

15   secretion and fluorescent labeling, prior to magnetic separation. Approximately 14.2% of the cells are in the positive window and are putative secretors. Figure 11c shows the fluorescence distribution of the non-magnetic fraction after magnetic separation. Nearly all positive

20   cells are retained in the magnetic column (2% of the cells in positive window). Figure 11d shows the fluorescence distribution of the magnetic fraction. The population of positive cells is highly enriched (80.3% in positive window). It should be noted that the purity of

25   the cell population can be expected to be higher than shown in the FACScan analysis because of instrument limitations. The enrichment rate can be calculated to greater than 24.

         Figures 10a to 10d show a similar experiment as

30   in figures 11a to 11d, except that a higher proportion of B1.8 to x63 cells was used. Medium during the secretion phase was RPMI containing 25% gelatin and 2.5% FCS. The percentage of cells in the positive window was 1.3% (control), 41.2% (after secretion), 6.6% (non-magnetic

35   fraction), and 92.9% (magnetic fraction). The enrichment

rate for positive cells in this example can be calculated
to be greater than 18.7, and the depletion rate greater
than 9.9.

Example 3

5   The following describes a method to measure the
absolute amount of secretion and to compensate for
different amounts of capture moiety on the cell surfaces.
        During the secretion phase the cells are
exposed to a low concentration of tagged product supplied
10   with the medium; the tagged product binds to but does not
saturate the product binding sites on the cells.
Incubation during this phase causes both the secreted
.product and the tagged product to bind to the cells. The
cells are then subjected to labeling with the label
15   moiety specific for the product (both tagged and
secreted). Measurement of the tag using one parameter,
and the total product in the other parameter, the amount
secreted by a cell is normalized, and the different
amounts of capture antibody on the cells in the mixture
20   is compensated for.

Example 4
Immunofluorescence analysis of live cells for secreted cytokines

        This example describes an immunofluorescence
25   method for the analysis of live cells for secreted
cytokines. More particularly, this example describes the
identification and separation of mouse spleen cells
secreting interferonγ (IFNγ). In general, this example
involves creating an artificial affinity matrix on the
30   surface of live cells by biotinylation of cell surface
proteins and incubation with an avidin-conjugated anti-
cytokine antibodies. The cells are incubated in a medium
of high viscosity to prevent diffusion of secreted
products between secreting and non-secreting cells and
35   allowed to secrete. Secreted cytokines caught on the

cell surface are labeled with digoxigenin (DIG)
conjugated anti-cytokine antibodies and stained using
fluorochromated anti-DIG antibodies. Cells labeled in
this manner can then be further characterized for surface
5   marker and sorted by MACS or FACS for functional assays.
Combining this method with the intracellular detection of
cytokines allows correlation of intracellular
accumulation and secretion of cytokines at the level of
single cells.
10

Detection of IFNγ-secreting murine spleen cells

          BALB/c mouse spleen cells (SC) were stimulated
with 2 μg/ml Staphylococcus aureus enterotoxin B (SEB;
Sigma) for about 40 hours at 2 x 10^6 cells/ml in RPMI
15   1640. The cells were then spun down for 10 min at 300 g.
The pellet was resuspended in 200 μl NHS-LC-biotin (1
mg/ml; Pierce) in PBS, pH 8.4, and then incubated for 15
min at room temperature. The cells were washed once with
PBS with 0.5% BSA (PBS/0.5%BSA), put in another tube and
20   washed a second time with PBS/0.5%BSA.
          The cells were resuspended in 200 μl
PBS/0.5%BSA with 0.02% NaN_3 (PBS/0.5%BSA/NaN_3) and
unconjugated anti-mouse IFNγ R46A2 (control) was added
until a final concentration of 10 μg/ml was achieved. In
25   the test samples avidin-conjugated anti-mouse IFNγ
AN18.17.24 was added to achieve a final concentration of
25 μg/ml. The cells were incubated for 5 min at 4°C.
Next, the cells were put into Petri dishes in 40% gelatin
(75 Bloom; Sigma) in RPMI 1640 (37°C) at 10^6 cells/ml for
30   10-60 minutes at 37°C and 7.5% CO_2.
          A 1.5 times volume of PBS (37°C) was added and
the cells put in a 50 ml tube with 2 volumes of PBS
(12°C). These cells were then spun down for 10 minutes
at 300 g. The pellet was resuspended in 100 μl DIG-
35   conjugated R46A2 (10 μg/ml) in PBS/0.5%BSA/NaN_3,

incubated for 10 min at 4°C, and then washed with
PBS/0.5%BSA/NaN$_3$. The pellet was next resuspended in 200
μl FITC conjugated sheep anti-DIG antibody (2 μg/ml) in
PBS/0.5%BSA/NaN$_3$, incubated for 10 minutes at 4°C, and

5   washed with PBS/0.5%BSA/NaN$_3$. The FACS analyses are
presented in Figures 13 and 14. Optionally, surface
labeling, fixation and intracellular labeling of the
cells can be performed and resuspended in
PBS/0.5%BSA/NaN$_3$.

10

Results

Secretion of IFNγ has been analyzed in mouse
spleen cells stimulated in vitro with SEB for 41 hours by
flow cytometry. Since IFNγ is produced only from large

15   activated cells (blasts), gating on live blasts was done
according to light scatter properties and propidium
iodide (PI) labeling (Figure 12). Biotinylation of cell
surface proteins was controlled by labeling with ST-FITC,
loading with the catching antibody, by labeling with FITC

20   conjugated goat anti-rat IgG (Figure 13). Figure 13a
depicts the distribution of unlabeled cells. Figure 13b
depicts the ability of ST-FITC to label cells before
biotinylation. Note that the cells are not
nonspecifically labeled by ST-FITC. Figure 13c depicts

25   the ability of ST-FITC to label biotinylated cells. Note
that the cells are completely labeled. Figure 13d
depicts labeling of cells that have not been labeled with
catch-ab with goat anti-rat IgG labeled with FITC
(GaRIgG-FITC). Figure 13d shows that there is no

30   nonspecific binding of the labeling antibody to the
cells. Figure 13e depicts labeling of cells labeled with
avidinated catch-ab with GaRIgG-FITC. Figure 13e
indicates that the catch-ab binds completely to the
cells.

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As negative control, cells without catch-ab
were incubated for 90 min in high density medium (HDM,
40% gelatine in RPMI) and labeled against IFNγ (Figure
14). As high control, cells with catch-ab incubated for
5   40 min in HDM were further incubated in IFNγ containing
supernatant for 10 min at 4°C and then labeled against
IFNγ (Figure 14). Among the SEB-stimulated murine SC,
labeled with catch-ab, an increasing number of IFNγ
secreting cells was detected after incubation in HDM
10   depending on the incubation time (Figure 14). Additional
surface labeling identified these IFNγ secreting SC as
CD4+ and CD8+ T cells. The SEB-stimulated T cell blasts
secrete varying amounts of IFNγ resulting in a wide
distribution of fluorescence intensity (Figure 14).
15   Figure 14 depicts the number of cells labeled
with αDIG-FITC under varying conditions. The αDIG-FITC
binds to the anti-IFNγ antibody coupled to DIG R46A2-DIG.
Figure 14a depicts the number of cells labeled in the
presence of catch-ab at zero time. Figures 14b, d and f
20   depict the number of cells labeled in the presence of
catch-ab after incubations of 5, 40 and 90 min. Note
that some cells have already been labeled and
increasingly more are labeled after 90 min. Figure 14c
depicts the number of cells labeled in the absence of
25   catch-ab after a 90-min incubation. Figure 14e depicts
the number of cells labeled with αDIG-FITC in the
presence of catch-ab and exogenous IFNγ added during the
incubation.

30   Industrial Utility
            The above-described methods and compositions
are useful for the detection and/or separation of cells
that secrete varying levels of one or more designated
substances. The cells may be phenotypically identical
35   except for their secretory activity of the designated

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product. Thus, the method may be of use in separating
cells that secrete commercially valuable substances from
those that do not, for example, cells that secrete
immunogenic polypeptides, growth factors, molecules that
5   can act as hormones, and a variety of other products,
including those produced by recombinant techniques. In
addition, the techniques may be useful in the isolation
of cell groups that are destined for transplantation or
implantation procedures, or for packaging for
10   implantation. Illustrative of this type of cell group
are the islets of Langerhans, where it would be desirable
to segregate groups of cells that are capable of
secreting insulin from those that are non-secretors. The
methods of determining the distribution of secretory
15   activity of cells in cell mixtures are also of use in
large scale fermentations in that they quickly identify
the appearance of nonsecretory or low secretory cell
variants or of cells producing a modified product.

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<u>Claims</u>

1. A method to separate cells according to a
product secreted and released by the cells wherein the
separation of cells is effected according to the degree
to which they are labeled with the product, the method
comprising the steps of:
    coupling the surface of the cells to a capture
moiety and culturing the cells under conditions wherein
the product is secreted, released and specifically bound
to the capture moiety; and
    separating the cells on the basis of the bound
product.

2. The method according to claim 1 further
comprising the step of labeling the product prior to
separation.

3. The method according to claim 2 wherein
the product is labeled with a label moiety.

4. The method according to claim 3 wherein
the label moiety is an antibody specific for the product.

5. The method according to claim 1 wherein the
label moiety is fluorochromated and the separation is
conducted by cell sorting.

6. The method according to claim 1 wherein the
label moiety is magnetizable and the separation is
conducted in a magnetic field of sufficient strength to
magnetized the label moiety.

7.   The method according to claim 6 wherein the label moiety comprises colloidal magnetic particles with a typical diameter of about 5 to 200 nm.

5   8.   The method according to claim 1 wherein the capture moiety is an antibody or an antigen-binding fragment thereof.

9.   The method according to claim 8 wherein
10   the antibody or antigen binding fragment thereof is bispecific.

10.   The method according to claim 1 wherein the coupling is through a lipid anchor attached to the
15   capture moiety optionally through a linking moiety.

11.   The method according to claim 1 wherein the coupling is through an antibody or an antigen-binding fragment thereof attached to the capture moiety,
20   optionally through a linker.

12.   The method according to claim 1 wherein the coupling is through direct chemical coupling of the capture moiety to components on the cell surface,
25   optionally through a linker.

13.   The method according to claim 9 wherein the coupling is through specific binding of the antibody to the cell.
30

14.   A method to label cells with a product secreted and released by the cells, which method comprises:
        coupling the surface of the cells to a capture
35   moiety;

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culturing the cells under conditions wherein
the product is secreted and released, wherein the product
is captured by the capture moiety.

5   15.   The method according to claim 14 wherein
the product is labeled with label moiety.

         16.   The method according to claim 15 wherein
the label moiety is an antibody.
10

         17.   The method according to claim 14 wherein
the specific binding partner is an antibody or an
antigen-binding fragment thereof.

15   18.   The method according to claim 17 wherein
the antibody is bispecific.

         19.   The method according to claim 14 wherein
the coupling is through a lipid anchor attached to the
20 specific binding partner optionally through a linking
moiety.

         20.   The method according to claim 14 wherein
the coupling is through an antibody or an antigen-binding
25 fragment thereof attached to the specific binding partner
optionally through a linker.

         21.   The method according to claim 18 wherein
the coupling is through specific binding of the antibody
30 to the cell.

         22.   A composition of matter which comprises
cells capable of capturing a product secreted and
released by the cells wherein the surface of the cells is
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coupled to a capture moiety, and wherein the specific
binding partner is not a hapten.

23. The composition according to claim 22
which is further coupled to the product.

24. The composition according to claim 22
wherein the capture moiety is an antibody or an antigen-
binding fragment thereof.

25. The composition according to claim 24
wherein the antibody is bispecific.

26. The composition according to claim 22
wherein the coupling is through a lipid anchor attached
to the capture moiety optionally through a linking
moiety.

27. The composition according to claim 22
wherein the coupling is through an antibody or an
antigen-binding fragment thereof attached to the capture
moiety, optionally through a linker.

28. The composition according to claim 25
wherein the coupling is through specific binding of the
antibody to the cell.

29. Cells and progeny thereof separated
according to the method of claim 1.

30. Cells separated according to the method of
claim 1.

31. A method of analyzing a population of
cells to identify or enumerate the cells that secrete an

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amount of product relative to other cells in the
population, the method comprising the steps of:

labeling the cells by the method according to
claim 14,

5   labeling the cells with at least one additional
label that does not label the captured product, and

detecting the amount of product label relative
to the additional label.

10   32. A method of determining a distribution of
secretory activity in a population of cells, the method
comprising the steps of:

labeling cells by the method according to claim
14, and

15   determining the amount of product label per
cell.

33. The method according to claim 14 further
comprising the steps of:

20   determining the amount and type of product
label per cell wherein distribution of secreted product
type and secretory activity for each secreted product
type in a population of cells is determined.

25   34. A kit for use in the detection of cells
that secrete a desired product, the kit comprising:

a product capture system comprised of at least
one anchor moiety and at least one capture moiety; and

at least one label moiety.
30

35. A kit for use in the detection of cells
that secrete a desired product, the kit comprising:

at least one bispecific antibody having at
least one antigen recognition site for at least one cell

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type and at least one antigen recognition site specific
for the product, and
at least one label moiety.

5   36.   The kit according to claim 35 wherein the
at least one bispecific antibody and the at least one
label moiety are in a single vial.

           37.   The kit according to claim 35 wherein the
10   at least one bispecific antibody binds to the cell
through a cell surface molecule.

           38.   The kit according to claim 37 wherein the
cell surface molecule is a naturally occurring cell
15   surface protein.

           39.   The kit according to claim 37 wherein the
cell surface molecule is a cell surface marker.

20   40.   The kit according to claim 39 wherein the
cell surface molecule is selected from the group
consisting of CD4, CD8, CD19, CD20, CD14, CD16, CD15,
CD45, Class I MHC molecules and Class II molecules, CD34,
CD38, CD33, CD56 T cell receptor, Fc receptor, β_2-
25   microglobulin, and immunoglobulin.

           41.   The kit according to claim 34 wherein the
incubation conditions include a high viscosity or gel
forming medium.
30
           42.   The kit according to claim 41 wherein the
medium is selected from the group consisting of gelatin,
agarose, alginate and combination thereof.

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43. The kit according to claim 37 wherein the label moiety is an antibody.

44. The kit according to claim 43 wherein the antibody comprises a detectable label.

45. The kit according to claim 44 wherein the detectable label is selected from the group consisting of fluorophores, radioactive isotopes, chromophores, and magnetic particles.

46. The kit according to claim 45 wherein the label moiety is detected by fluorescence activated cell sorting.

47. The kit according to claim 37 wherein the label moiety is detected by a third antibody.

48. The kit according to claim 47 wherein the label moiety is coupled to digoxigenin and the third antibody is specific for digoxigenin.

49. The kit according to claim 47 wherein the third antibody comprises a detectable label.

50. The kit according to claim 35 further comprising a biological modifier.

51. The kit according to claim 35 further comprising a cell-cell cross-contamination reducing capture system.

52. A method for identifying cells secreting product comprising the steps of:

combining a mixed population of cells with at least one first, bispecific, antibody, each antibody, having combining sites specific for a cell surface molecule and at least one product;

5   incubating the combination under conditions and for a time sufficient to allow the cells to secrete the at least one product;

adding at least one label moiety; and

detecting the at least one label moiety.

10

53.   The method according to claim 52 further comprising the step of separating the cells secreting the product from the mixed cell population.

15   54.   The method according to claim 53 wherein the cell surface molecule is a naturally occurring cell surface protein.

55.   The method according to claim 54 wherein

20   the protein is a cell surface marker.

56.   The method according to claim 55 wherein the cell surface marker is selected from the group consisting of CD4, CD8, CD19, CD20, CD14, CD16, CD15,

25   CD45, Class I MHC molecules and Class II MHC molecules, CD34, CD38, CD33, CD56, T cell receptor, Fc receptor, β_2-microglobulin, and immunoglobulin.

57.   The method according to claim 52 wherein

30   the incubation conditions include a high viscosity or gel forming medium.

58.   The method according to claim 52 wherein the label moiety is an antibody.

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59. The method according to claim 58 wherein the antibody comprises a detectable label.

60. The method according to claim 59 wherein the label is selected from the group consisting of fluorophores, radioactive isotopes, chromophores, and magnetic particles.

61. The method according to claim 60 wherein the label moiety is detected by fluorescence activated cell sorting.

62. The method according to claim 61 wherein the label moiety is detected by a third antibody.

63. The method according to claim 62 wherein the label moiety is coupled to digoxigenin and the third antibody is specific for digoxigenin.

64. The method according to claim 62 wherein the third antibody comprises a detectable label.

65. The method according to claim 64 wherein the label is selected from the group consisting of fluorophores, radioactive isotopes, chromophores, and magnetic particles.

66. The method according to claim 65 wherein the label moiety is detected by fluorescence activated cell sorting.

67. The method according to claim 52 wherein the label moiety comprises a magnetizable moiety.

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68.   The method according to claim 67 wherein
the label moiety is detected by a third antibody coupled
to a magnetizable moiety.

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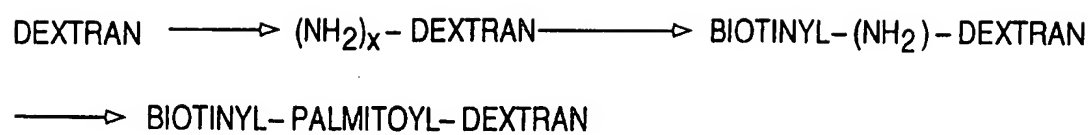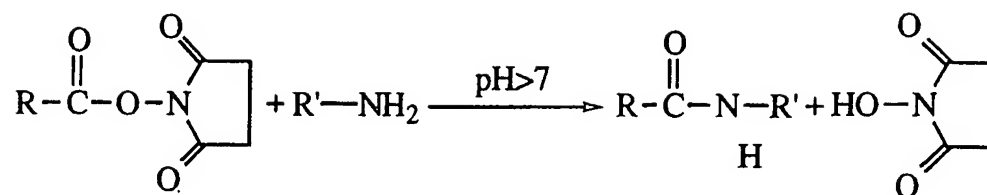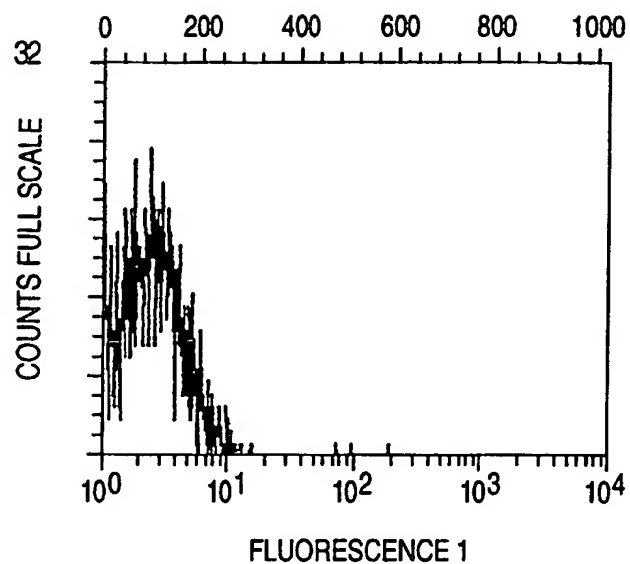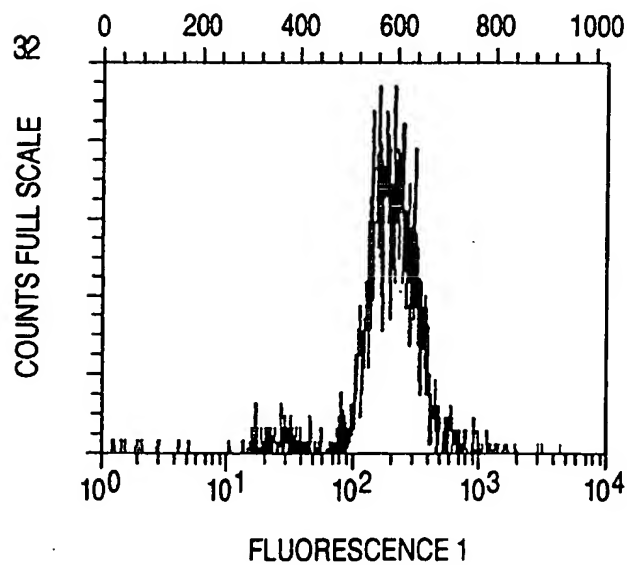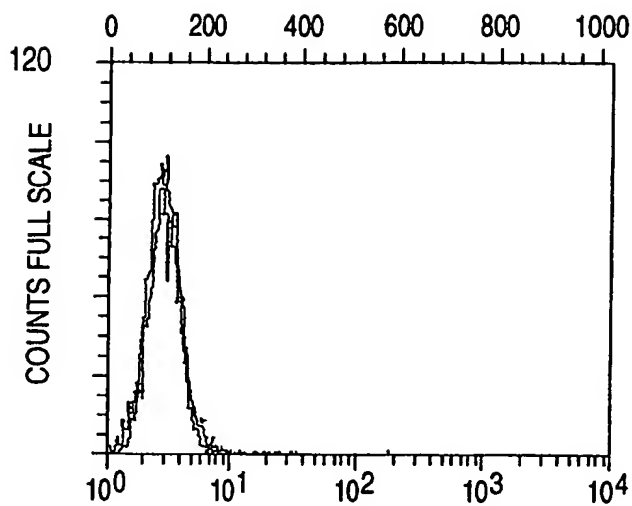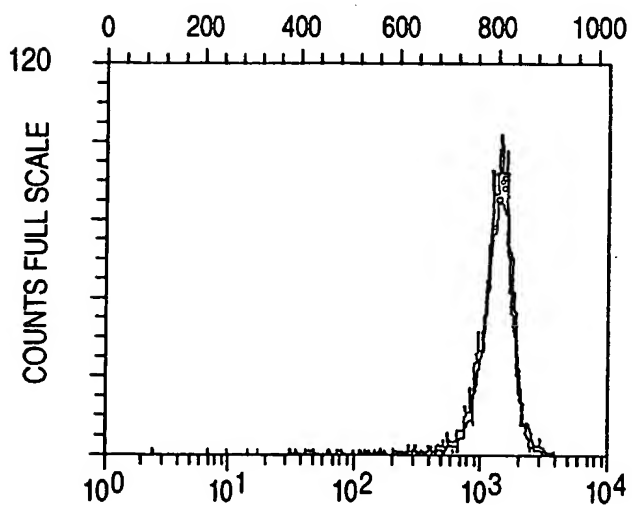
DEXTRAN $\longrightarrow$ $(NH_2)_x$ – DEXTRAN $\longrightarrow$ BIOTINYL – (NH_2) – DEXTRAN

$\longrightarrow$ BIOTINYL – PALMITOYL – DEXTRAN

FIG. 1

FIG. 2

FIG. 3a

FIG. 3b

FIG. 4a

FIG. 4b
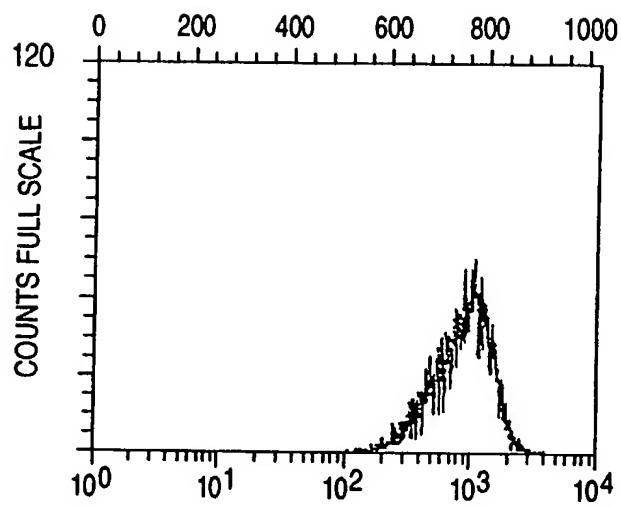
FIG. 4c
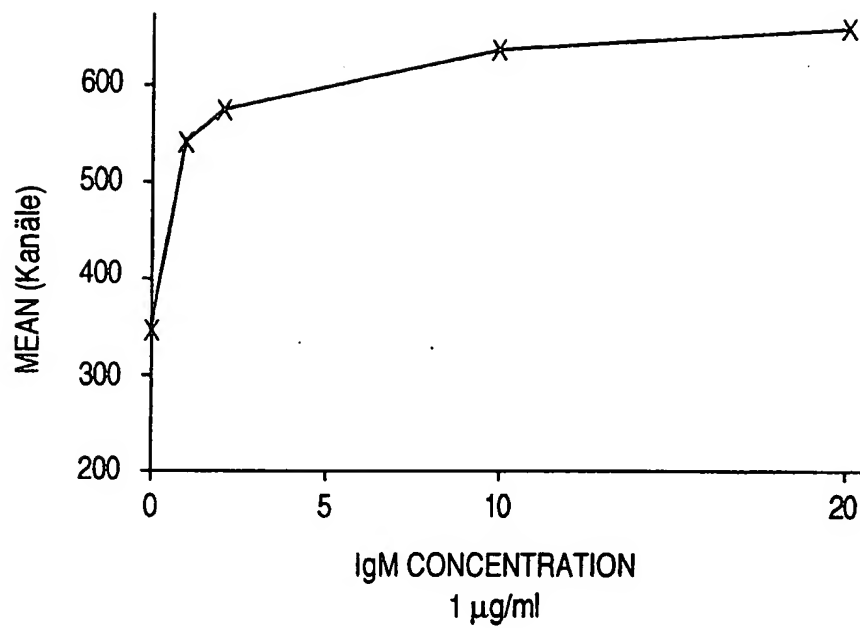
FIG. 5
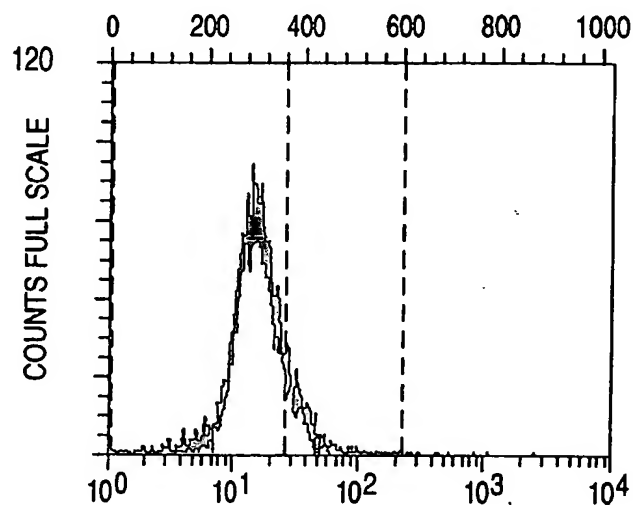
FIG. 6a
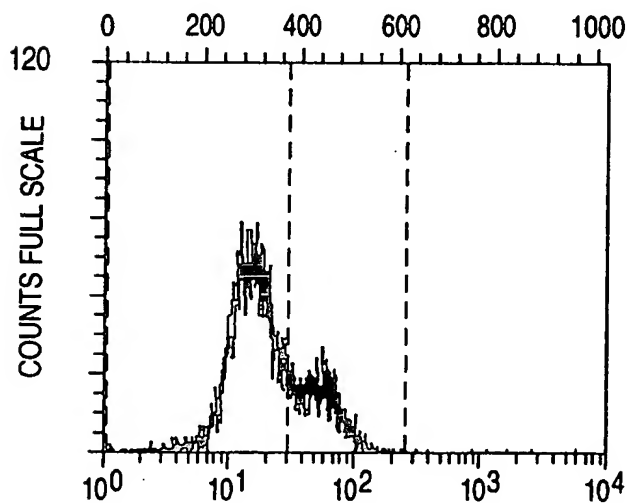
FIG. 6b
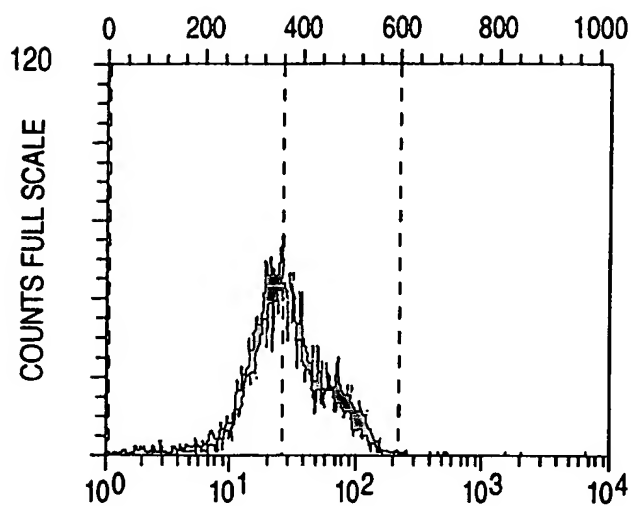
FIG. 6c
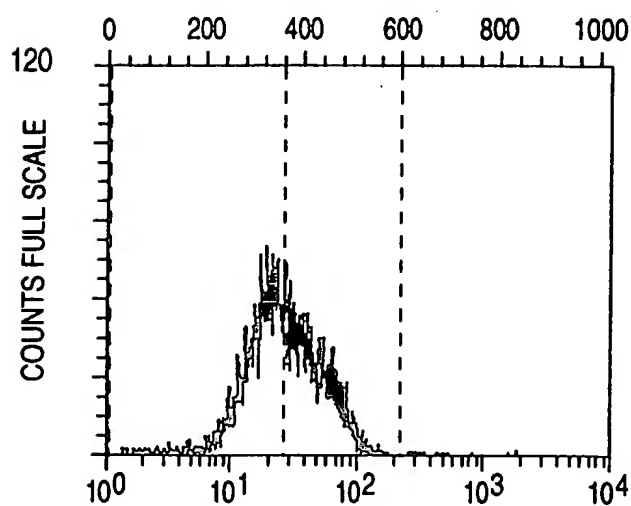
FIG. 6d
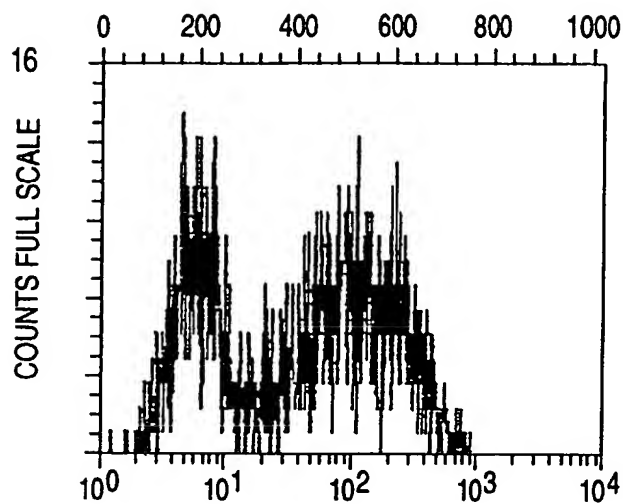
FIG. 7a
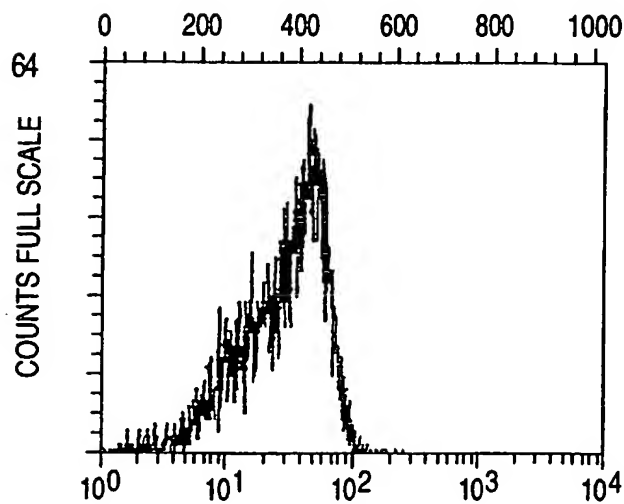
FIG. 7b
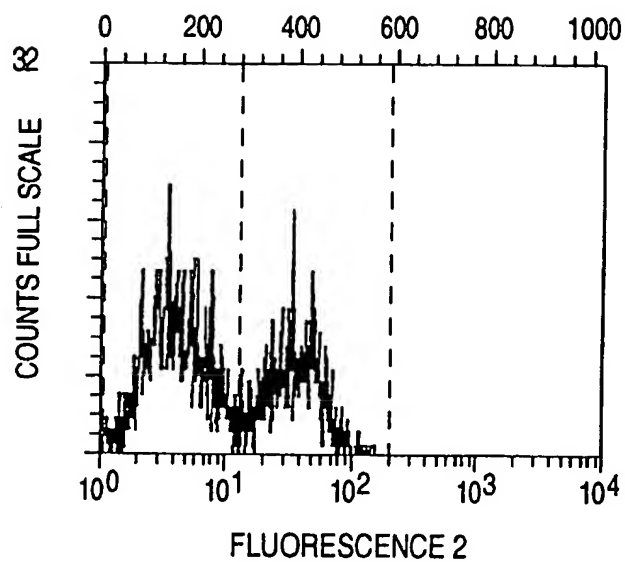
FIG. 8a
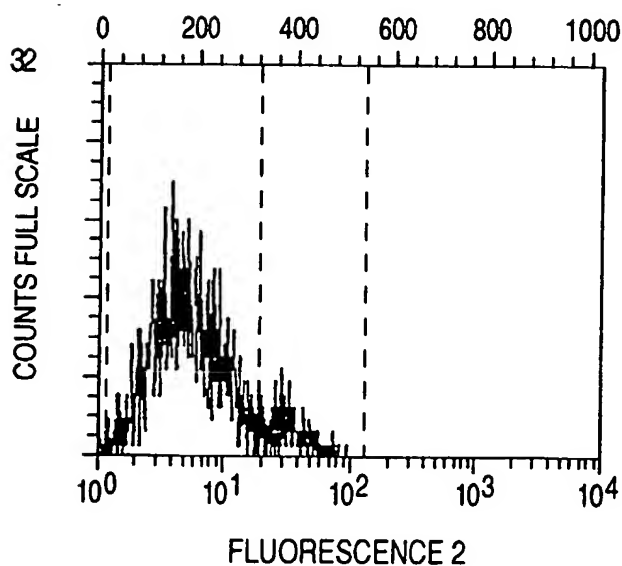
FIG. 8b
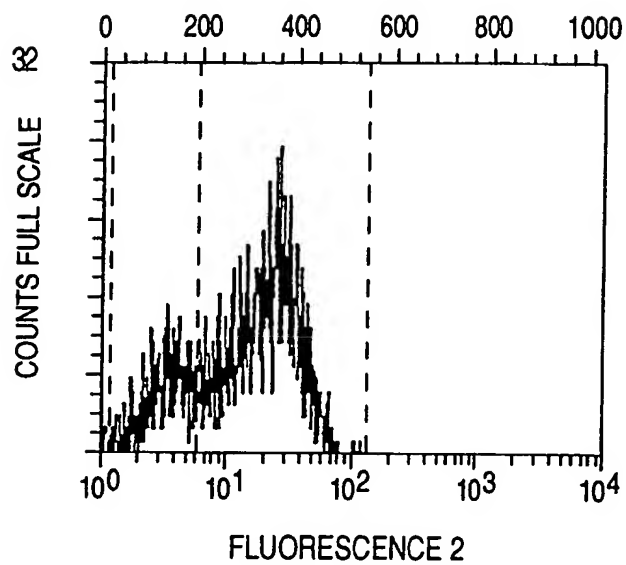
FIG. 8c
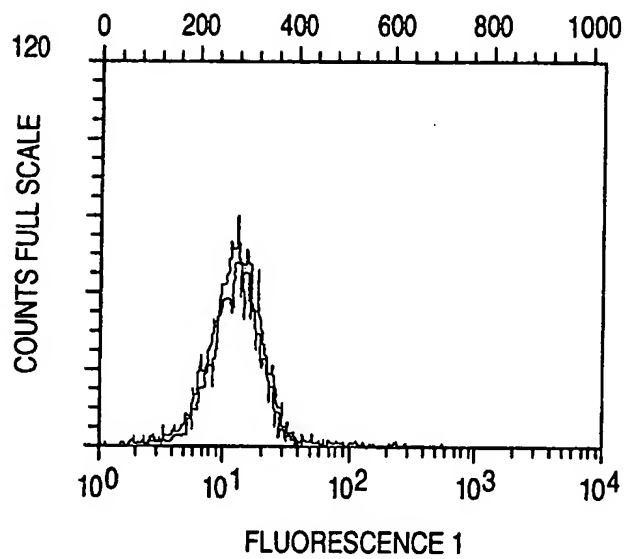
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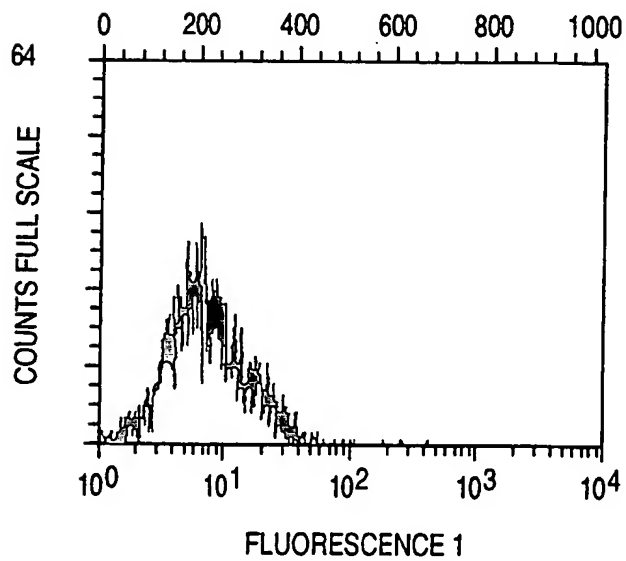
FIG. 9b

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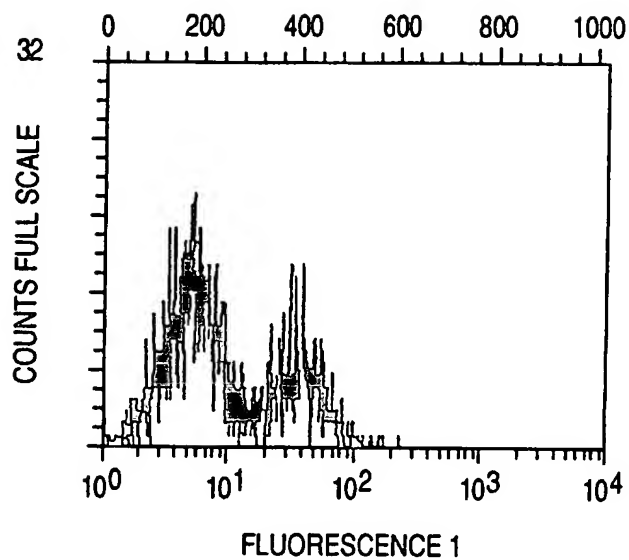
FIG. 9c
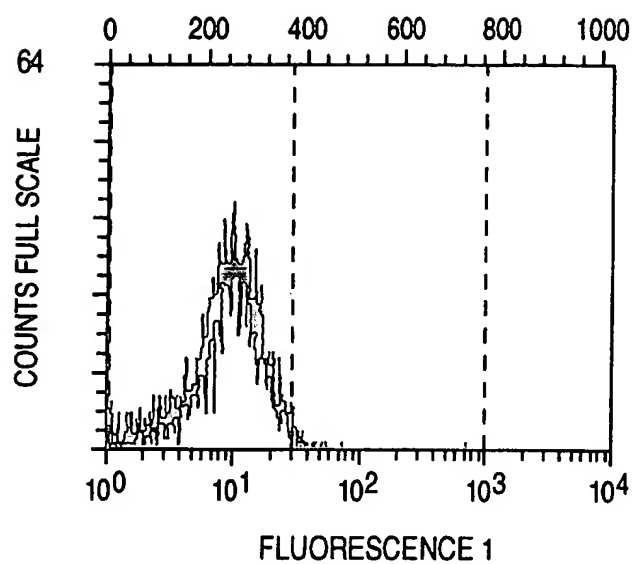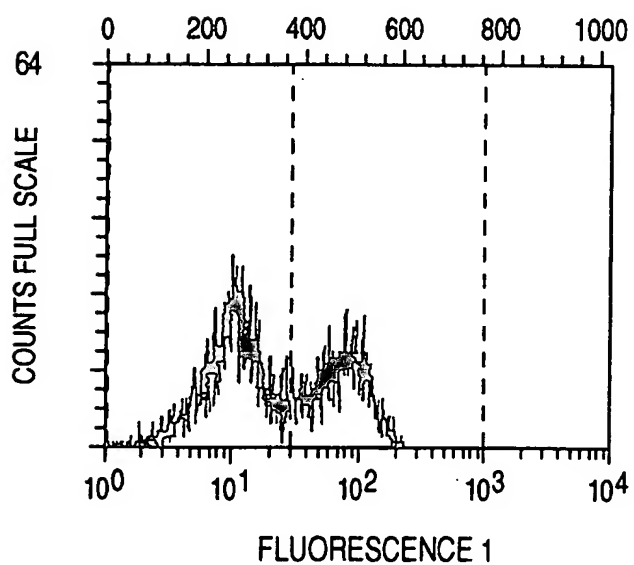
FIG. 10a

FIG. 10b

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FIG. 10c
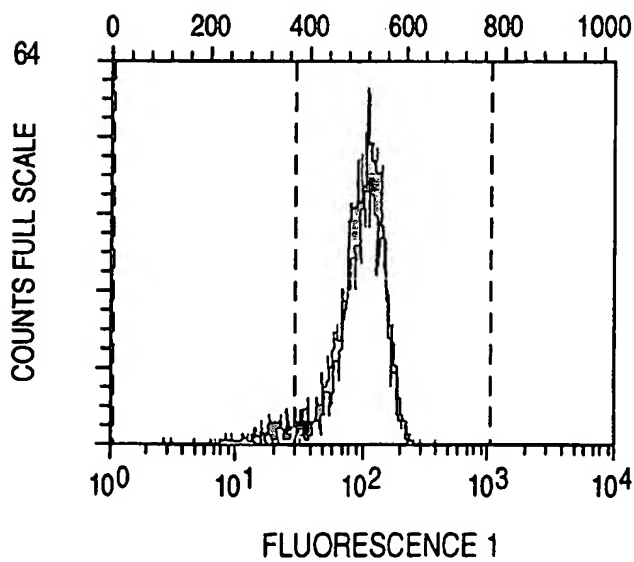
FIG. 10d
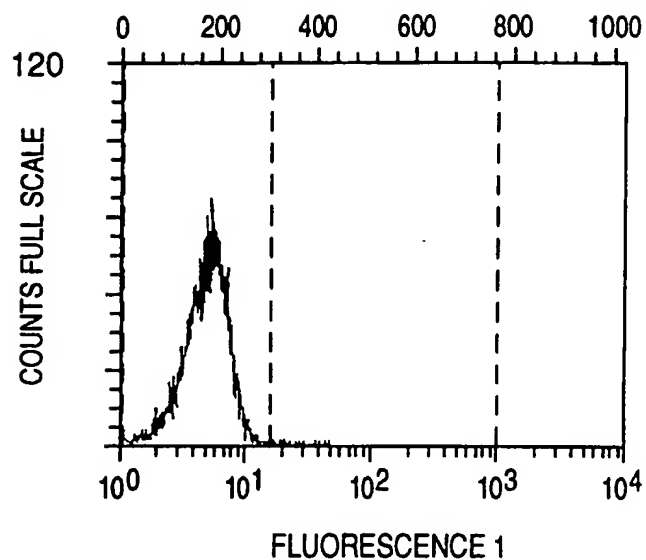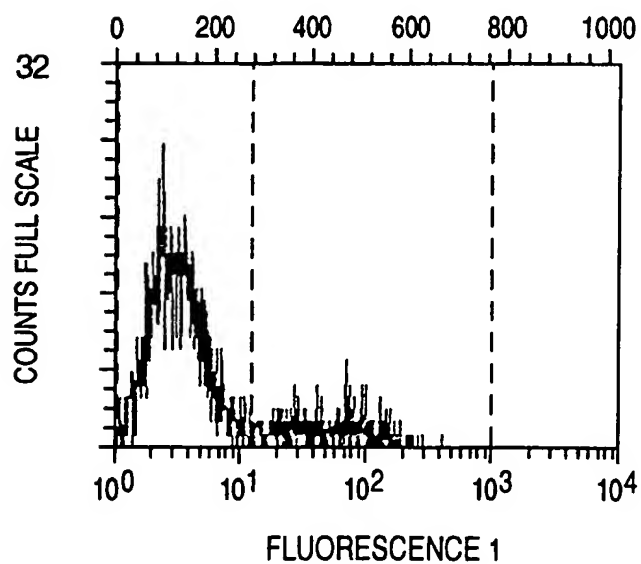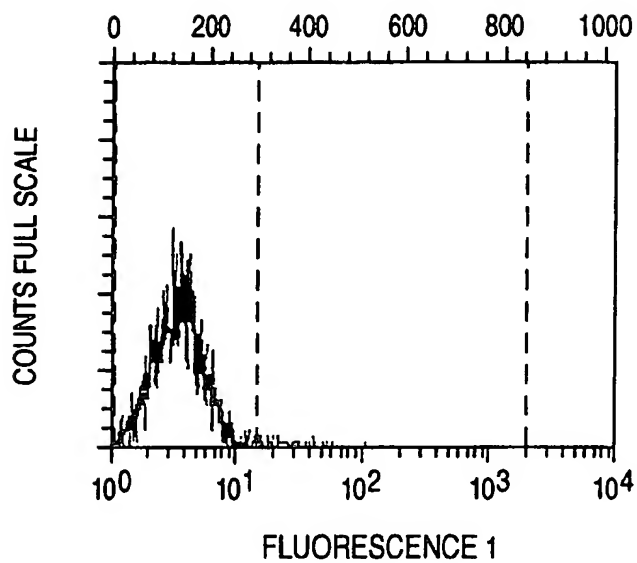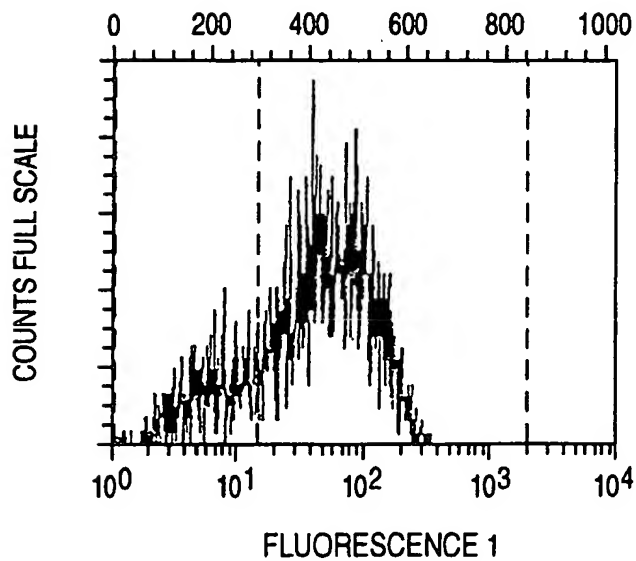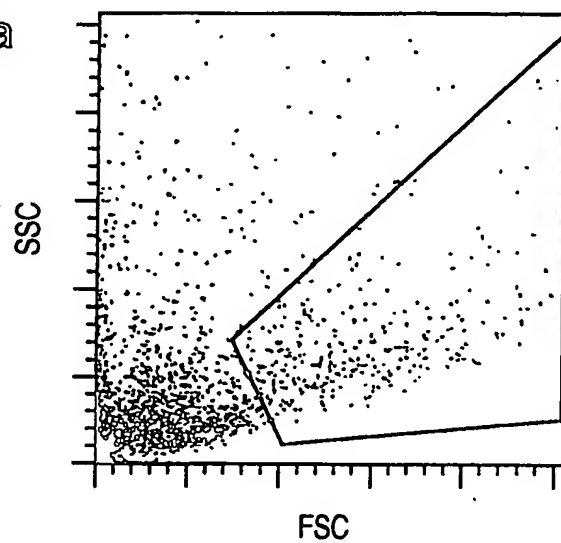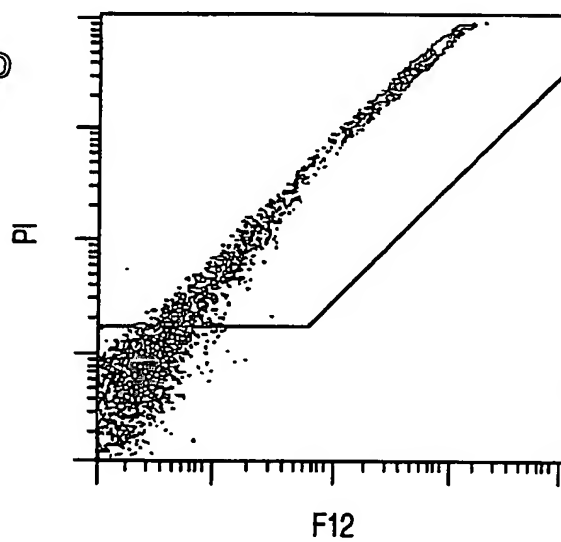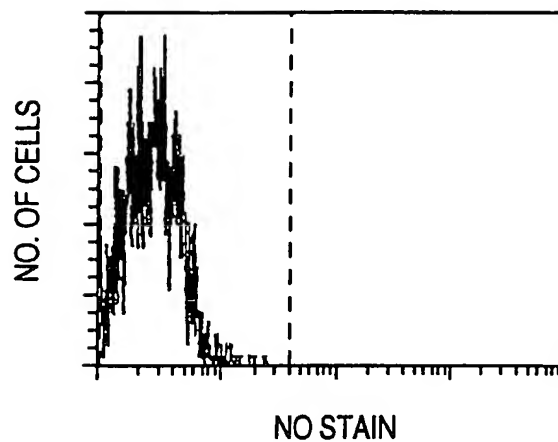
FIG. 11a

FIG. 11b

FIG. 11c

FIG. 11d

FIG. 12a

SSC

FSC

FIG. 12b

PI

F12

FIG. 13a

NO STAIN

FIG. 13b

ST - FITC

FIG. 13c

ST - FITC

FIG. 13d

NO. OF CELLS

GaRIgG - FITC

FIG. 13e

NO. OF CELLS

GaRIgG - FITC

FIG. 14a

FIG. 14b

IFNγ
(R46A2-DIG αDIG-FITC)

FIG. 14c

NO CATCH ab
90' INCUBATION

FIG. 14d

NO CATCH ab
40' INCUBATION

NO STAIN

IFNγ
(R46A2-DIG αDIG-FITC)

FIG. 14e

FIG. 14f

CATCH ab
IFNγ sup

CATCH ab
90' INCUBATION

NO STAIN

IFNγ
(R46A2-DIG αDIG-FITC)
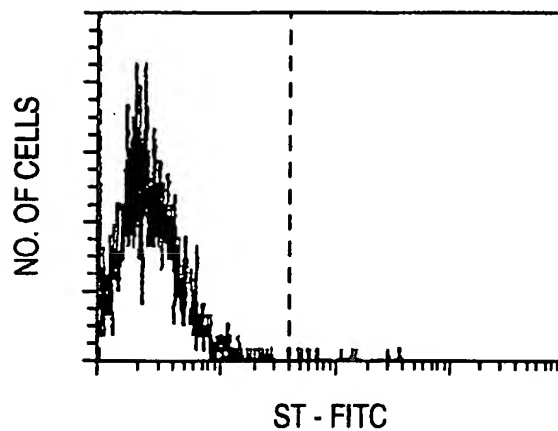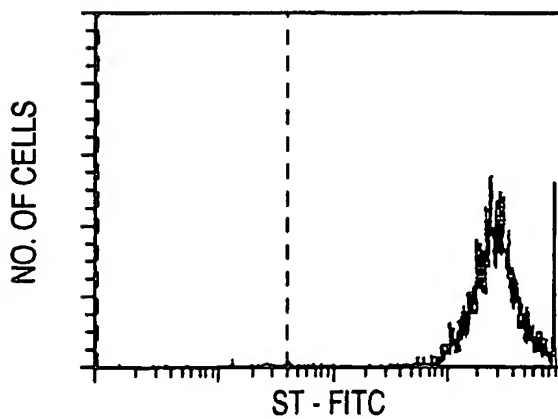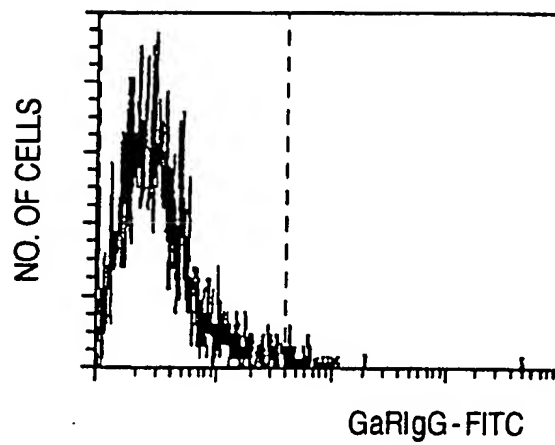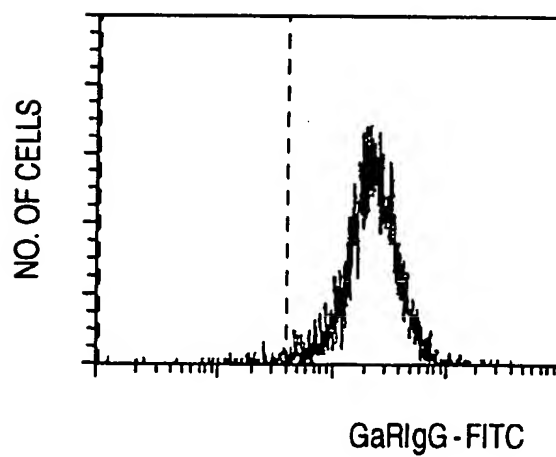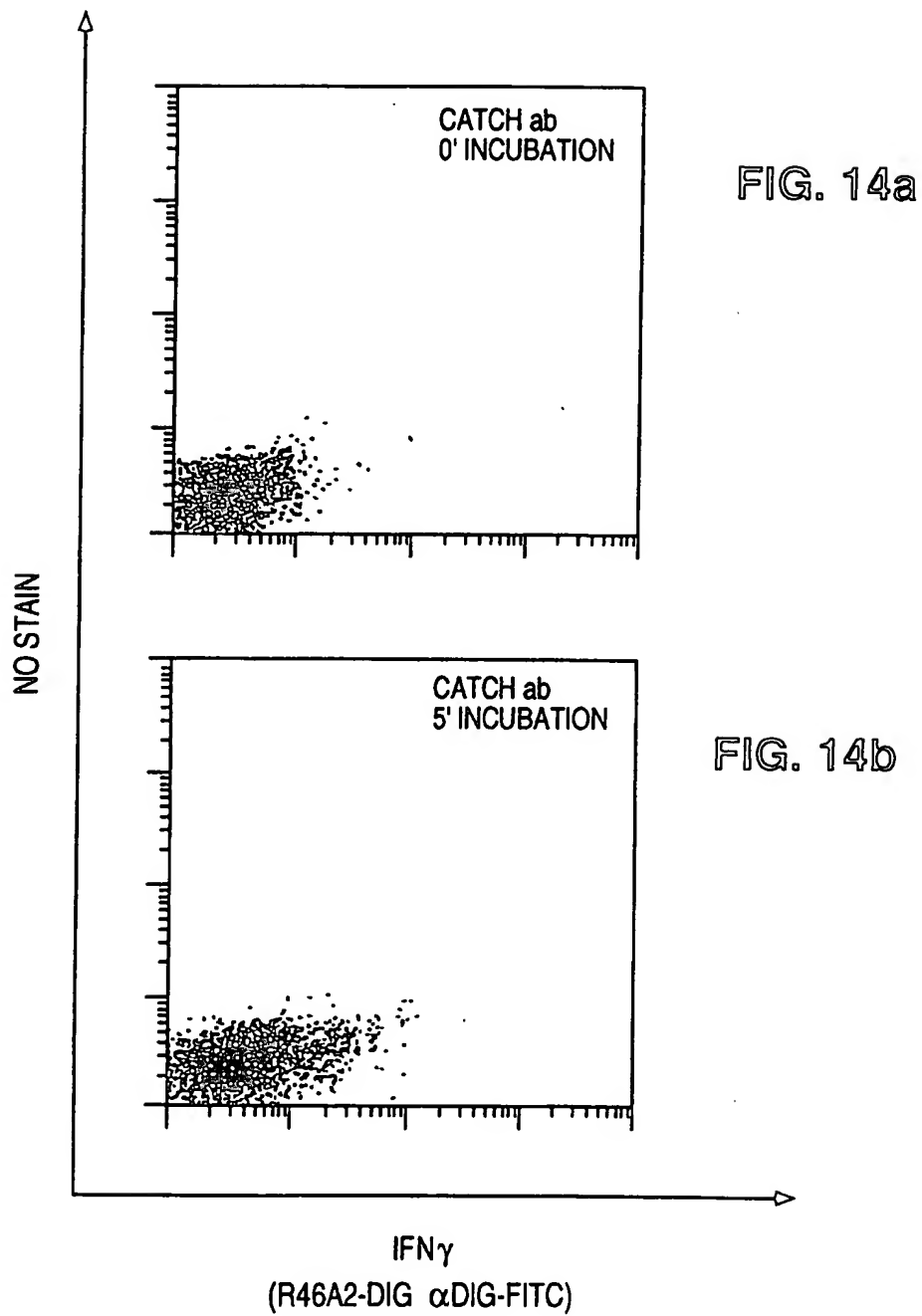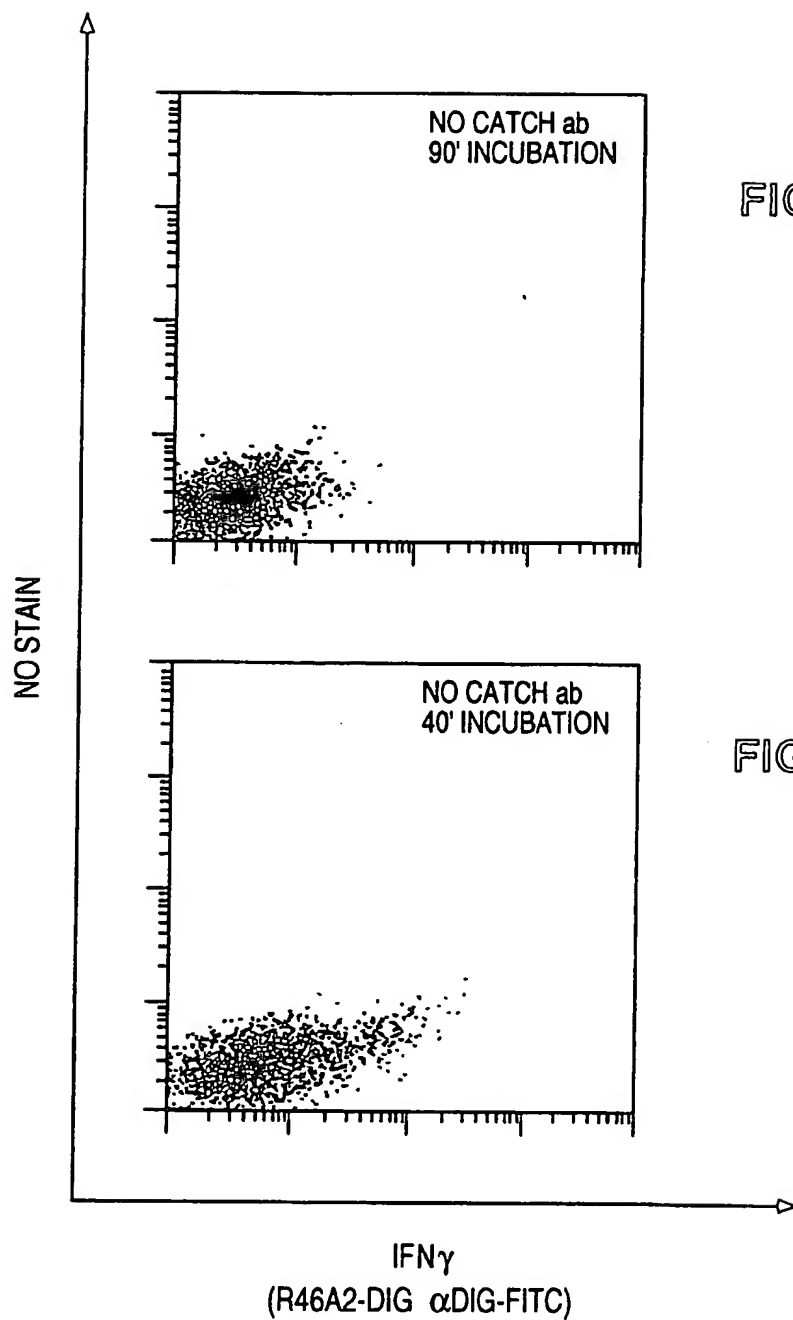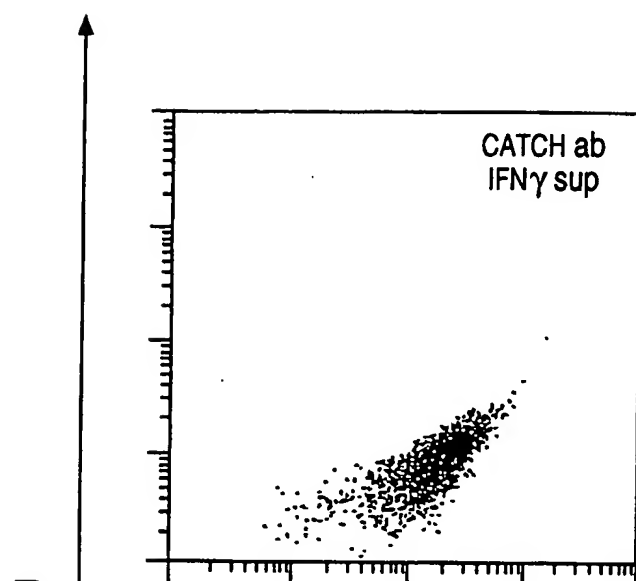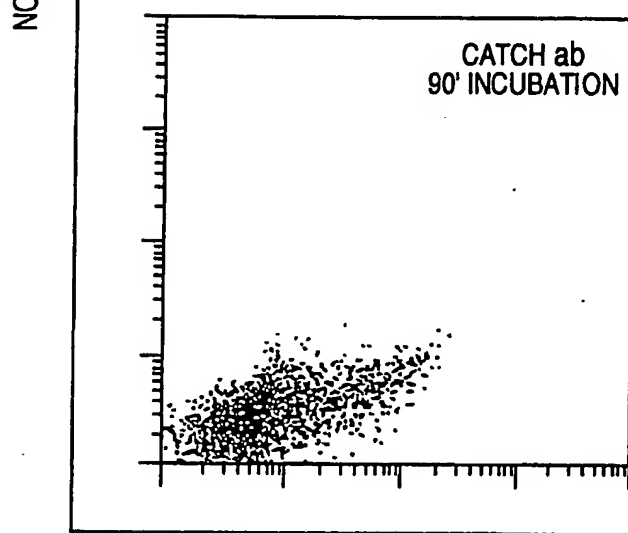
INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL   :435/2, 7.2, 7.21, 240.2; 436/535; 530/391.1, 391.9
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/2, 7.2, 7.21, 240.2; 436/535; 530/391.1, 391.9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proceedings National Academy Sciences USA, Volume 76, Number 4, issued April 1979, D.R. Parks et al., "Antigen-specific identification and cloning of hybridomas with a fluorescence-activated cell sorter", pages 1962-1966, see entire document.	29,30
X	Proceedings National Academy Sciences USA, issued March 1986, A.M. Weissman et al., "Only high-affinity receptors for interleukin 2 mediate internalization of ligand", pages 1463-1466, see entire document.	22,23

[X] Further documents are listed in the continuation of Box C.	[ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 JANUARY 1994	0 2 FEB 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	RON SCHWADRON
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.
PCT/US93/10126

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	European Journal of Immunology, Volume 10, issued October 1980, G. Kohler et al., "Immunoglobulin M mutants", pages 467-476, see entire document.	1-21,24-28,31-68

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12N 5/00; A01N 1/02; G01N 33/536, 33/58, 33/566, 33/53, 33/563; C12Q 1/00, 1/02

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Biosis, Medline, Chem Ab, Embase, Derwent WPI, APS, search terms: bivalent antibody, antigen capture, bifunctional antibody, secretion, antibody, surface labelled, coupled, linked, attached, cell, bacteria, assay, immunoassay, cell surface, membrane, lymphokine, cell separation